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Identification of *Shigella* Invasion Genes by Isolation of Temperature-Regulated *inv*::*lacZ* Operon Fusions

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Penetration and multiplication within cells of the human colonic epithelium are hallmarks of *Shigella* spp. pathogenicity. *Shigella* spp. virulence is regulated by growth temperature. Strains phenotypically virulent when grown at 37°C are phenotypically avirulent when grown at 30°C. The number of genes involved in *Shigella* spp. pathogenicity and how many virulence genes are temperature regulated are unknown. To facilitate the study of temperature-regulated virulence in *Shigella* spp., we employed *lacZ* operon fusion technology to identify temperature-regulated invasion (*inv*) genes. Four *inv*::*lacZ* fusion mutants were identified and found to be unable to invade HeLa cells. The fusions were located in a region of the 220-kilobase invasion plasmid defined as the minimal amount of DNA required for invasion, and they were controlled by *virR*, the temperature-dependent virulence gene regulator. Western blot (immunoblot) and Southern hybridization analyses indicated that one of the fusions was located in a known *inv* gene, *ipaB*, which encodes one of the major immunogenic peptides of *Shigella* spp. This *ipaB*::*lacZ* operon fusion mutant synthesized a truncated IpaB protein recognized by IpaB-specific monoclonal antibodies. Three of the fusions were within novel genes mapping to regions previously identified as essential for a positive virulence phenotype. Analysis of bacterial surface proteins suggested that the genes marked by these fusions may play a role in the correct surface expression of the *ipaB* and *ipaC* gene products.

Shigella spp. are enteric pathogens of humans whose pathogenicity is characterized by their ability to penetrate and multiply within cells of the colonic epithelium (12). The process of invasion and intracellular multiplication leads to epithelial cell death, inflammation, and ulcerative lesions of the colon resulting in the characteristic disease symptoms of severe dysentery and/or diarrhea. Several assay systems are employed to distinguish the different steps in *Shigella* spp. virulence. Included among these are the Séreny test, which measures infection and destruction of mucosal surfaces resulting in keratoconjunctivitis in guinea pigs (26); the plaque assay, which measures intracellular replication and intercellular spread (22); and the HeLa cell invasion assay (10). The use of these various assay systems and the application of classical genetic techniques have demonstrated that *Shigella* spp. virulence is a multigenic phenomenon. Genes encoded on the 220-kilobase (kb) invasion plasmid (25) and several unlinked chromosomal loci (24) are essential for the expression of a complete virulence phenotype. In addition, expression of *Shigella* spp. virulence is regulated by growth temperature. *Shigella* strains which are phenotypically virulent when cultured at 37°C become phenotypically avirulent when cultured at 30°C (15). *virR*, a gene required for the temperature-dependent regulation of Shigella spp. virulence, has been identified and cloned (18). Current knowledge of *Shigella* virulence and its regulation is, however, limited, as it is not known precisely how many plasmid and chromosomal genes are involved in the pathogenicity of *Shigella* spp. Similarly, the number of these virulence genes which are temperature regulated is not known.

In order to address these issues and facilitate the study of the temperature regulation of *Shigella* spp. virulence, we employed the *lac* operon fusion system of Bremer et al. (5) to identify temperature-regulated virulence genes. Random insertions of the transposable bacteriophage λp*lac*Mu53 in a virulent strain of *S. flexneri* 2a can be screened for positive *lacZ* operon fusions which (i) cause loss of virulence, thereby identifying virulence genes; and (ii) display temperature-dependent expression of β-galactosidase, indicating operon fusions to temperature-regulated promoters. By employing this system, we identified four virulence genes involved in *Shigella* spp. invasion, as well as three non-virulence-associated genes, each of which is regulated by growth temperature. Three of these noninvasive mutants appeared to be altered in surface expression of proteins essential for *Shigella* invasion.

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MATERIALS AND METHODS

Bacterial strains and cultivation. The bacterial strains used are described in Table 1. Bacterial strains were routinely cultured in LB broth (8) or tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) at either 37 or 30°C. Antibiotics were added to broth cultures or agar media at the following concentrations: ampicillin, 30 μg/ml; kanamycin, 50 μg/ml; tetracycline, 5 μg/ml. TSB agar medium was prepared by adding Bacto-Agar (Difco) to TSB at a final concentration of 1.5%. Congo red (Sigma Chemical Co., St. Louis, Mo.) was added to TSB agar at 0.025% in order to determine dye binding.

Mutagenesis by λp*lac*Mu53 and screening for *inv*::*lacZ* operon fusions. Random insertions of the transposable phage λp*lac*Mu53 were isolated in strain BS181 as previously described (5). BS181 was infected with λp*lac*Mu53 in the presence of helper phage λpMu507. Lactose-fermenting (Lac^+), kanamycin-resistant (Km^r) transductants were selected at 37°C on M9 salts minimal medium (8) supplemented with lactose (0.5%), kanamycin, and nicotinic acid (10 μg/

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TABLE 1. Bacterial strains

Strain	Relevant genotype or description[a]	Reference or source
E. coli K-12 DH5α	φ80d Δ(*lacZYA-argF*) *hsdR17* (r_K^- m_K^-)	BRL[b]
S. flexneri 2a		
2457T	Wild type harboring 220-kb invasion plasmid pSf2a140	9
BS103	2457T cured of pSf2a140	16
BS181	2457T Mal$^+$ λ^s	18
BS189	2457T *virR1*::Tn*10*	18
BS226	2457T pSf2a140 Φ(*mxi*::*lacZ*$^+$ 11.5)	This study
BS228	2457T pSf2a140 Φ(*ipaB*::*lacZ*$^+$ 17.6)	This study
BS230	2457T pSf2a140 Φ(*mxi*::*lacZ*$^+$ 17.7)	This study
BS232	2457T pSf2a140 Φ(*mxi*::*lacZ*$^+$ 18.15)	This study
BS234	2457T pSf2a140 Φ(*lacZ*$^+$ 17.9)	This study
BS236	2457T pSf2a140 Φ(*lacZ*$^+$ 18.11)	This study
BS238	2457T pSf2a140 Φ(*lacZ*$^+$ 18.13)	This study

[a] Φ indicates presence of an operon fusion to the gene indicated; all operon fusions shown contain an insert of the fusion bacteriophage λp*lac*Mu53.
[b] BRL, Bethesda Research Laboratories, Inc.

ml) and screened for temperature regulation of lactose utilization as determined by growth at 37°C and no growth at 30°C. Mutants which expressed a temperature-regulated Lac$^+$ phenotype were screened for loss of virulence in the HeLa cell invasion assay. All temperature-regulated λp*lac*Mu53 operon fusions were then transduced into the strain 2457T background by generalized transduction, using bacteriophage P1 *vir* as described by Silhavy et al. (27). Lac$^+$ Km^r transductants were detected on LB agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and further characterized for alteration of their virulence phenotypes.

Virulence assays. Virulence properties of *S. flexneri* strains were measured by HeLa cell invasion (10), plaque formation in HeLa cell monolayers (22), and production of keratoconjunctivitis in guinea pigs in the Séreny test (26). All samples were assayed in triplicate. Contact hemolytic activity was determined by the method previously described by Clerc et al. (7). Mutants which are noninvasive in the HeLa cell assay are both plaque assay and Séreny test negative.

β-Galactosidase assay. β-Galactosidase activity was measured by the method of Miller (19). Bacterial cultures were grown overnight at 30 and 37°C in TSB and then subcultured at the same temperatures and allowed to grow approximately four to five generations before being assayed for β-galactosidase. Enzyme assays were performed at room temperature on chloroform–sodium dodecyl sulfate-permeabilized bacteria.

Plasmid preparation and agarose gel electrophoresis. Rapid plasmid screening of 1.5 ml of overnight cultures was done by the alkaline lysis procedure described by Birnboim and Doly (4). Large-scale plasmid DNA isolation was performed by the same method, and plasmid DNA was further purified by cesium chloride-ethidium bromide density gradient centrifugation. DNA was electrophoresed in horizontal 0.7% agarose gels in Tris-acetate buffer (13).

Western blotting (immunoblotting). Whole-bacterium extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters (Bio-Rad Laboratories, Richmond, Calif.) as described by Burnette (6). The nitrocellulose filters were prepared for treatment with primary antibody as previously described (20). Convalescent-phase serum (diluted 1:100) from a human who had been infected with *S. flexneri* 2a was used to detect expression of the four major antigenic polypeptides encoded by the virulence plasmid, pSf2a140, of strain 2457T and the *lacZ* operon fusion mutants. Monoclonal antibodies (MAbs) 2F1, 1H4, 4C8, and 5H1, isolated and characterized by Mills et al. (20), were used to specifically identify two of the four major antigenic polypeptides produced by *Shigella* spp. Following exposure to the primary antibody, filters was washed and incubated with alkaline phosphatase-conjugated staphylococcal protein A (Kirkegaard and Perry Laboratories, Gaithersburg, Md.), followed by exposure to the phosphatase substrate as described previously (20).

Preparation of DNA probes and Southern hybridization. DNA probes were prepared by restriction digestion of plasmid DNA followed by isolation and purification of DNA fragments from agarose gels by electroelution (Elutrap; Schleicher & Schuell, Inc., Keene, N.H.). Whole-plasmid or restriction fragment probes were labeled with ^{32}P (Amersham Corp., Arlington Heights, Ill.) by random primer labeling (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.). DNA fragments were transferred from agarose gels to nitrocellulose filters (Bio-Rad), and hybridizations were carried out by the method of Southern (28).

Cloning of *inv*::*lacZ* fusions. Each of the *inv*::*lacZ* fusions was cloned by using the *lacZ* fusion cloning vector pMLB524 (3). Plasmid DNA prepared from each *inv*::*lacZ* fusion mutant was digested with *Eco*RI and ligated to *Eco*RI-digested and alkaline phosphatase-treated pMLB524. Cloned DNA was transformed into *Escherichia coli* DH5α by the technique described by Hanahan (11). Positive clones were selected on medium containing ampicillin and X-Gal.

ELISA. The whole-cell enzyme-linked immunosorbent assay (ELISA) was performed by a procedure previously described (20). MAb 2F1 or 2G2, at a dilution of 1:250, was added as the primary antibody to bacterium-coated 96-well polystyrene microtiter plate wells (Costar, Cambridge, Mass.). Goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) at a 1:100 dilution was used as the secondary antibody label. Each bacterial sample was tested in quadruplicate.

RESULTS

Isolation and initial characterization of temperature-regulated *lacZ* operon fusions. The invasive *S. flexneri* strain BS181 was infected with bacteriophage λp*lac*Mu53, and lysogens expressing Km^r, the selective marker encoded by the transposing phage, were selected for the ability to utilize lactose (Lac$^+$) at 37°C. Approximately 10,000 Lac$^+$ Km^r lysogens were subsequently screened for the Lac$^+$ phenotype at 37 and 30°C. Of the 10,000 Lac$^+$ mutants screened, 7 independently isolated mutants were temperature regulated for lactose utilization in that they expressed the Lac$^+$ phenotype only at 37°C. The actual levels of β-galactosidase expressed by these mutants at 37 and 30°C were determined. In each case, β-galactosidase expression at 37°C was at least fivefold greater than at 30°C (data not shown). After establishing that the seven fusion mutants isolated were the result of *lacZ* fusions to temperature-regulated genes, each mutant was tested for expression of virulence in the HeLa cell invasion assay. Four of the seven mutants exhibited a complete loss of invasion ability (Inv$^-$), while the other three expressed an invasion phenotype equivalent to that of the parental strain, BS181. Preliminary characterization of the temperature-regulated *lacZ* fusion mutants indicated, as

TABLE 2. Virulence phenotypes of temperature-regulated *lacZ* operon fusion mutants

Strain	HeLa cell invasion[a]	Plaque assay[b]	Séreny test[c]	Contact hemolysis (%)[d]	Congo red binding		*virR1* control[e]
					30°C	37°C	
BS226	−	ND[f]	ND	3	−	−	+
BS228	−	$<1 \times 10^{-8}$	−	7	−	+	+
BS230	−	ND	ND	9	−	−	+
BS232	−	ND	ND	15	−	−	+
BS234	++++	7.2×10^{-4}	+	80	−	+	+
BS236	++++	6.2×10^{-4}	+	106	−	+	−
BS238	++++	5.3×10^{-4}	+	104	−	+	+
2457T	++++	6.3×10^{-4}	+	100	−	+	+
BS103	−	$<1 \times 10^{-8}$	−	16	−	−	NA[g]

[a] Data represent percentage of HeLa cells invaded: (number of HeLa cells invaded/total number of HeLa cells counted) × 100. ++++, 77 to 100%; +++, 54 to 76%; ++, 31 to 53%; +, 10 to 30%; − <10%. Cells containing ≥5 bacteria were considered invaded.

[b] Plaquing efficiency expressed as number of plaques per input CFU.

[c] Positive Séreny test was determined to be elicitation of keratoconjuntivitis 48 h postinoculation.

[d] Values represent percentage of mean absorbance obtained for wild-type strain 2457T.

[e] See Table 3 for β-galactosidase 37-to-30°C ratios.

[f] ND, Not determined.

[g] NA, Not applicable.

TABLE 3. Expression of β-galactosidase by *inv::lacZ* operon fusion mutants and their *virR*::Tn*10* derivatives[a]

Strain	U of β-galactosidase[b]		Ratio, 37°C/30°C	U of β-galactosidase[b] (*virR*::Tn*10*)		Ratio, 37°C/30°C
	37°C	30°C		37°C	30°C	
BS226	550	11	50	1,080	580	1.9
BS228	382	6.3	60	840	210	4.0
BS230	668	14	49	1,730	560	3.0
BS232	468	4.4	106	1,390	440	3.2
BS234	132	43	3	98	204	0.5
BS236	194	40	5	312	104	3.0
BS238	338	54	6	180	160	1.1

[a] Values presented are based on representative experiments.

[b] Units of β-galactosidase are as defined by Miller (19).

previously shown (17), that two separate populations of *lacZ* operon fusions could be isolated, one in which the genes marked by the fusion were involved in the invasion of HeLa cells (*inv::lacZ*) and a second in which the invasion phenotype was apparently unaltered.

Further characterization of the operon fusion mutants was performed after transduction of each fusion from the mutagenized strain into 2457T, a nonmutagenized wild-type strain. The resultant Lac^+ Km^r transductants were tested both for temperature-regulated expression of β-galactosidase and for the ability to invade HeLa cells. Each transductant tested expressed the same phenotype as the original operon fusion mutant from which it was derived (Table 2). Fusion strains BS226, BS228, BS230, and BS232 were Inv^-, while fusion mutants BS234, BS236, and BS238 remained Inv^+; all seven mutants expressed a 37-to-30°C β-galactosidase ratio greater than or equal to 3. These results confirmed that the original mutant strains were each the result of a single-site insertion of the transposing bacteriophage and thus, for the noninvasive mutants, this marked genes which were involved in the process of invasion. These data also demonstrated that the two populations of fusions isolated were also different in their relative expression of β-galactosidase at 37 and 30°C. Each noninvasive operon fusion mutant showed a 37-to-30°C β-galactosidase ratio greater than 40. In marked contrast, the mutants which remained invasive all expressed β-galactosidase ratios of less than 10 (Table 3).

Phenotypic characterization of invasive *lacZ* operon fusion mutants. (i) Plaque and Séreny assays. Although three of the *lacZ* operon fusion mutants were still invasive for HeLa cells, this virulence assay defines only one step in *Shigella* pathogenicity. In order to determine whether another step in *Shigella* virulence may have been altered as a result of mutation by λp*lac*Mu53, the three Inv^+ operon fusion isolates were screened for virulence as measured by the plaque and Séreny assays. The results (Table 2) indicate that the fusion mutants were identical to the wild-type strain 2457T in their ability to form plaques and cause a positive Séreny test. Keratoconjunctivitis elicited by the fusion mutants and 2457T developed after 48 h, and the intensities were equivalent.

(ii) Ability of *lacZ* operon fusion mutants to bind Congo red dye. Temperature-regulated binding of Congo red is characteristic of virulent *Shigella* strains, and loss of Congo red binding (Pcr^-) is accompanied by loss of invasion ability (16). Genetically, the Pcr^- phenotype has been attributed to a complete loss of, or deletions in, the 220-kb invasion plasmid (16). The seven operon fusion mutants were plated on medium containing Congo red dye and incubated at 37 and 30°C to determine the effect of the *lacZ* operon fusions on Congo red binding. All of the invasive mutants as well as one of the noninvasive mutants, BS228, bound Congo red only at 37°C, exhibiting a Pcr^+ phenotype and colonial morphology identical to that of 2457T. The Inv^- mutants BS226, BS230, and BS232 exhibited a Pcr^- phenotype at both 37 and 30°C corresponding to the Pcr phenotype of Inv^- strains.

(iii) Contact hemolytic activity. Although *S. flexneri* classically is defined as nonhemolytic, the organism has been shown to lyse sheep erythrocytes when in close contact with them (7). This phenotype has been shown to correlate strongly with several different virulence traits, notably invasion. The *lacZ* fusion mutants were tested for contact hemolytic activity relative to the parental strain 2457T and its derivative BS103, cured of the invasion plasmid. The *inv::lacZ* fusion mutants all expressed an Hly^- (contact hemolytic activity negative) phenotype (Table 2). The percentage of contact hemolytic activity of the *inv::lacZ* mutants was less than or equal to 15% of the activity expressed by wild-type strain 2457T and comparable to that of the Hly^- control strain BS103 (16%). In contrast, the invasive *lacZ* operon fusion mutants were as efficient in their ability to induce contact hemolysis as 2457T.

***virR* control of temperature-regulated *lacZ* operon fusions.** *virR* has been identified as a gene required for temperature-dependent regulation of *Shigella* virulence (18). Each of the *lacZ* operon fusion mutants was screened for loss of temperature-regulated β-galactosidase expression in the presence of a *virR*::Tn*10* mutation. A P1 *vir* lysate made on *S. flexneri* 2a strain BS189 was used to transduce a *virR*::Tn*10* mutation into the *lacZ* operon fusion mutants. The ratios of β-galactosidase activity at 37 versus 30°C in the *inv::lacZ* strains (BS226, BS228, BS230, and BS232) were markedly reduced after introduction of the *virR* mutation (Table 3). In the presence of the wild-type *virR* gene, each of these strains had a 37-to-30°C β-galactosidase ratio greater than 40, while in the presence of a *virR*::Tn*10* mutation, the ratio was reduced to a value less than or equal to 5. Decreased

β-galactosidase ratios were largely due to increased enzyme levels at 30°C, and therefore these data indicate a deregulation of β-galactosidase expression in the *inv*::*lacZ virR*:: Tn*10* strains relative to the parental strains harboring a wild-type *virR* gene. The temperature-regulated *lacZ* fusion mutants BS234 and BS238 exhibited a similar reduction of the 37-to-30°C β-galactosidase ratio. Ratios of 3 (BS234) and 6 (BS238) decreased to ratios less than 1 when a *virR* mutation was present. Fusion mutant BS236, however, showed no significant reduction in the β-galactosidase ratio in the presence of a *virR* mutation.

Localization of the temperature-regulated *inv*::*lacZ* operon fusions by Western blot analysis. Random insertion of λp*lac*Mu53 in the mutant strains may result in the *inv*::*lacZ* operon fusions being located on either the chromosome or the 220-kb invasion plasmid. If inserted into the 220-kb invasion plasmid, the λp*lac*Mu53 genome should be detectable by agarose gel electrophoresis since its large size (approximately 50 kb) would retard the migration of the plasmid in the gel. The first step in localizing the temperature-regulated *inv*::*lacZ* fusions involved a comparison of the plasmid profiles of the *inv*::*lacZ* operon fusion strains with that of 2457T. The 220-kb invasion plasmids in the four noninvasive operon fusion mutants were larger than the wild-type pSf2a140 (data not shown). This indicated that each of the *inv*::*lacZ* operon fusions was located on the 220-kb invasion plasmid. Use of this same technique showed that each of the Inv$^+$ operon fusions also mapped to the invasion plasmid.

We attempted to more precisely locate the Inv$^-$ fusions on the plasmid by Western blot analysis. Previous studies have shown that a 37-kb fragment of the 220-kb invasion plasmid defines the minimum sequence necessary to confer an Inv$^+$ phenotype on an invasion plasmid-less derivative of *S. flexneri* (14). In addition, the invasion plasmid antigen genes (*ipa*) are located within this 37-kb subclone (pHS4108). The *ipa* genes encode the four major antigenic polypeptides, IpaA (70 kilodaltons [kDa]), IpaB (62 kDa), IpaC (48 kDa), and IpaD (32 kDa), detected by post-*Shigella* infection convalescent-phase human and monkey sera (21). Moreover, Tn*5* insertions in various regions of pHS4108, including *ipaB*, lead to a knockout or reduction of the invasion-positive phenotype (2). In order to localize the *inv*::*lacZ* operon fusions on pSf2a140 and determine if the fusions inactivated any one of the *ipa* genes, the operon fusion mutants were analyzed by Western blot, using both IpaA-, IpaB-, IpaC-, and IpaD-specific polyclonal sera and MAbs specific for IpaB and IpaC. Fusion mutants BS226, BS230, and BS232 were identical to 2457T in their Ipa protein profile (Fig. 1A, lane 2). Fusion mutant BS228, however, had an altered Ipa protein profile, with the bands representing IpaA, IpaC, and IpaD no longer visible and the band migrating at the same position as IpaB having an increased staining intensity. This finding suggests that of the four *inv*::*lacZ* operon fusion mutants isolated, only BS228 had an operon fusion located in the *ipa* coding region.

The recent isolation and characterization of MAbs to IpaB and IpaC (20) provided us with the opportunity to further characterize the location of the *lacZ* operon fusion in strain BS228. Western blot analysis using the IpaC-specific MAb, 5H1, showed no detectable IpaC production by BS228 (Fig. 1C). In contrast, all of the IpaB-specific MAbs (2F1, 1H4, and 4C8) detected a protein in the BS228 lysate of considerably smaller molecular weight than that of wild-type IpaB (Fig. 1B). These results are evidence that the *lacZ* operon fusion in BS228 is located in the *ipaB* coding region and as a result of the insertion the strain synthesized a truncated IpaB product. Binding by the IpaB-specific MAbs also indicated that the site of the fusion end joint was between *lacZ* and *ipaB* in BS228. Each of the IpaB MAbs used bind different epitopes located in the amino terminus of the IpaB protein which are encoded for by a DNA sequence of approximately 800 base pairs. This coding sequence represents a polypeptide of approximately 28 to 30 kDa (20). The fusion end joint in BS228 can therefore be localized to a point approximately 1 kb 3′ from the previously determined transcriptional start site of *ipaB*.

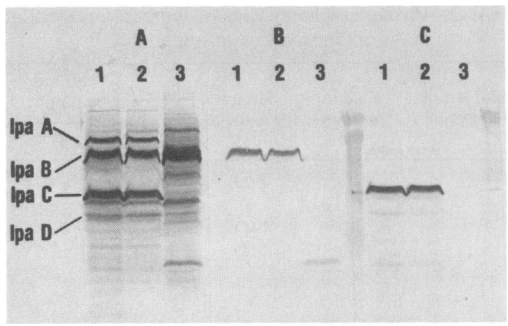

FIG. 1. Western blot of whole-cell bacterial lysates probed with convalescent-phase human polyclonal antisera (1:100 dilution) from a patient infected with *S. flexneri* 2a (A), MAb 2F1 (1:300 dilution) (B), or MAb 5H1 (1:300 dilution) (C). Lanes: 1, 2457T; 2, BS226, BS230, and BS232; 3, BS228.

Localization of the temperature-regulated *inv*::*lacZ* operon fusions by Southern hybridization. Four subclones spanning the entire length of pHS4108 were used as probes to map the location of the *inv*::*lacZ* fusions by Southern hybridization (Fig. 2). The fusion end joints of the different *inv*::*lacZ* fusions were first cloned into the *lacZ* fusion cloning vector pMLB524 (3). pMLB524 contains 309 base pairs of the *lac* operon coding region starting at the unique *Eco*RI site in *lacZ* and codes for the 17 carboxy-terminal amino acids of β-galactosidase. The plasmid alone displays a LacZ$^-$ phenotype. By cloning *Eco*RI fragments from a *lacZ* fusion mutant into this vector, the *lacZ* gene is regenerated and a LacZ$^+$ phenotype results. The bacterial DNA represented in the cloned insert includes DNA from the fusion end joint to the next *Eco*RI site 5′ to *lacZ*. *Eco*RI restriction of each fusion end joint subclone generated two fragments representing the cloning vector and the *Eco*RI fusion end joint insert (Fig. 3A). The sizes of the cloned *Eco*RI fragments from each of the *inv*::*lacZ* fusions ranged from 5 to 14 kb. After subtraction of 3 kb to account for the *lacZ* portion of the cloned fragments, the bacterial DNA represented in the subcloned fusion fragments had the following sizes: fusion 11.5 (BS226), 10 kb; fusion 17.6 (BS228), 2.0 kb; fusion 17.7 (BS230), 4.0 kb; fusion 18.15 (BS232), 7.0 kb. Southern hybridization with pHS4108 subclones as probes (Fig. 2) indicated that each of the *Eco*RI insert subclones hybridized to pHS5103 (Fig. 3C). In addition, the *Eco*RI insert fragment of the fusion 11.5 subclone hybridized to pHS5101 (Fig. 3B). None of the fusion end joint subclones hybridized to pHS5102 or pHS5500 (data not shown). These data, in addition to generally localizing the temperature-regulated *inv*::*lacZ* fusions on pHS4108, corroborate the location of the fusion in BS228 (*ipaB*::*lacZ*$^+$ 17.6) as determined by Western blot analysis.

In an attempt to determine more specifically the location of the two temperature-regulated *inv*::*lacZ* fusions, 17.7 (BS230) and 18.15 (BS232), subcloned fusion end joints of

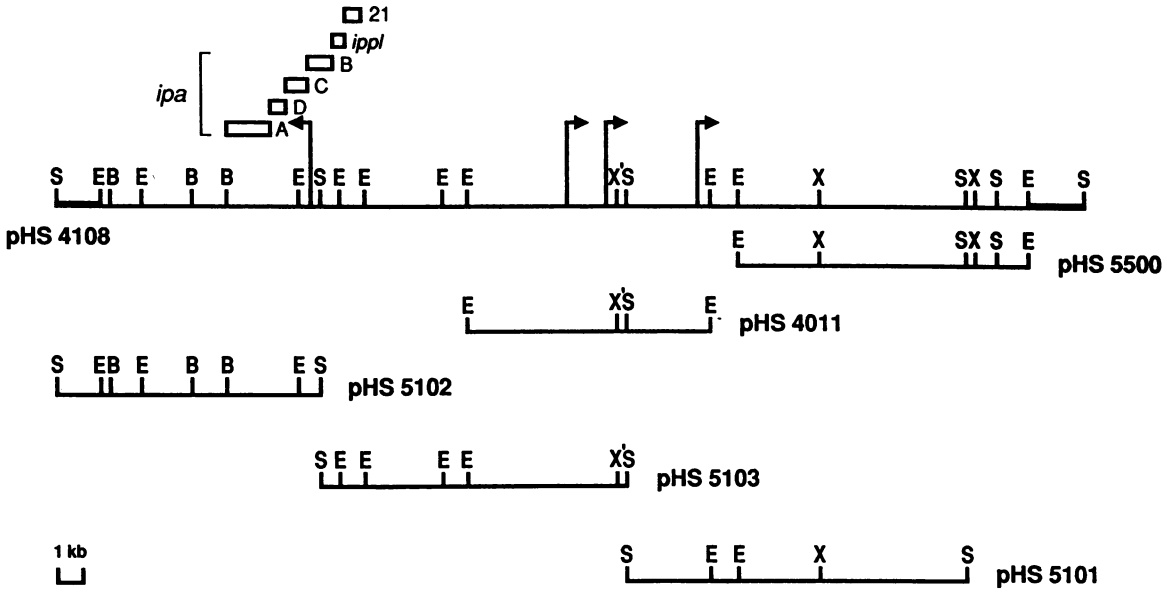

FIG. 2. Partial restriction maps of recombinant plasmid pHS4108 (2) and pHS4108 subclones used as probes in Southern hybridization of *inv*::*lacZ* subclones (Fig. 3). Thick lines represent the cloning vector; ↰ and ↱ represent *lacZ* operon fusion insertions (from left to right, BS228, BS230, BS232, and BS226), with the arrows denoting the directions of transcription of the fusions. E, B, X, X′, and S represent the restriction sites of *Eco*RI, *Bam*HI, *Xba*I, *Xho*I, and *Sal*I, respectively.

each were hybridized with the *Eco*RI fragment from pHS4011, which spans the junction between subclones pHS5101 and pHS5103 (Fig. 2). Both fusion subclones hybridized to the *Eco*RI fragment of pHS4011 (Fig. 3D), thereby localizing the fusion end joints for strains BS230 and BS232 to the 8-kb *Eco*RI-*Sal*I fragment of pHS4108 (Fig. 2). In addition to the site of the insertion, we can also conclude on the basis of the hybridization data that the direction of transcription of the fusion is opposite to that of the *ipa* operon.

Southern hybridization analysis (Fig. 3D) indicated that fusion 11.5 (BS226) was also located on the 11-kb pHS4011 *Eco*RI insert. On the basis of further restriction digest and hybridization analyses, using pHS5101 and pHS5103 as probes, we determined that the cloned insert portion of the fusion 11.5 subclone represented DNA from the unique *Eco*RI site located in the *lacZ* coding sequence to a *Hin*dIII site approximately 1 kb 5′ to the 11.5 fusion end joint. The end joint for fusion 11.5 was therefore precisely mapped to a site located approximately 0.5 kb to the left of the *Eco*RI site indicated (Fig. 2), and we concluded that transcription was in the same direction as that of fusions in strains BS230 and BS232.

Detection of surface IpaB and IpaC by whole-cell ELISA. A previous study by Mills et al. (20) employing MAbs 2F1 and 2G2 in a whole-cell ELISA system led to the conclusion that IpaB and IpaC are exposed on the surface of the bacterium. Our Western blot data indicated that each of the Inv$^-$ mutants expressed one or all of the Ipa proteins intracellularly. We sought to determine by whole-cell ELISA whether the Inv$^-$ phenotypes of our *inv*::*lacZ* fusion mutants may be due to the altered surface expression of IpaB and IpaC.

The results obtained with MAb 2F1 (Table 4) indicate that the expression of the truncated IpaB in BS228 was approximately equivalent to that of the wild-type IpaB in the positive control strain 2457T. The truncated IpaB produced by the fusion mutant appears therefore to contain sufficient sequence information to be processed and expressed on the bacterial surface. BS226, BS230, and BS232 expressed reduced levels of IpaB on their surfaces.

On the basis of the results obtained with MAb 2G2 (Table 4), mutant strain BS228 appeared to express a low level of surface IpaC. This appears to contradict the results of Western blot analysis, which indicated that BS228 expressed no IpaC. It is possible, however, that given the range of sensitivities for these two assay systems, Western blotting may not have detected low-level expression of IpaC. With MAb 2G2, BS226, BS230, and BS232 showed intermediate surface expression of IpaC. The decreased expression of these protein products on the surfaces of the bacteria may explain the Inv$^-$ phenotype observed for all four of these fusion mutant strains.

DISCUSSION

Studies characterizing *Shigella* spp. virulence and its regulation have provided evidence that this multigenic trait is dependent on the expression of chromosomal and plasmid-encoded virulence determinants (24, 25), resulting in a

TABLE 4. Expression of surface IpaB and IpaC on Inv$^-$ temperature-regulated *lacZ* fusion mutants

Strain	A_{405}[a]	
	MAb 2F1[b]	MAb 2G2[c]
2457T	1.174 ± 0.104	1.889 ± 0.024
BS226	0.249 ± 0.025	0.700 ± 0.064
BS228	0.882 ± 0.037	0.111 ± 0.003
BS230	0.247 ± 0.035	0.680 ± 0.065
BS232	0.243 ± 0.014	0.613 ± 0.047
BS103	0.046 ± 0.020	0.048 ± 0.003

[a] Representative experimental values expressed as means of quadruplicate samples ± standard deviations of the means. A_{405} values ≤0.1 were considered negative.
[b] IpaB-specific MAb.
[c] IpaC-specific MAb.

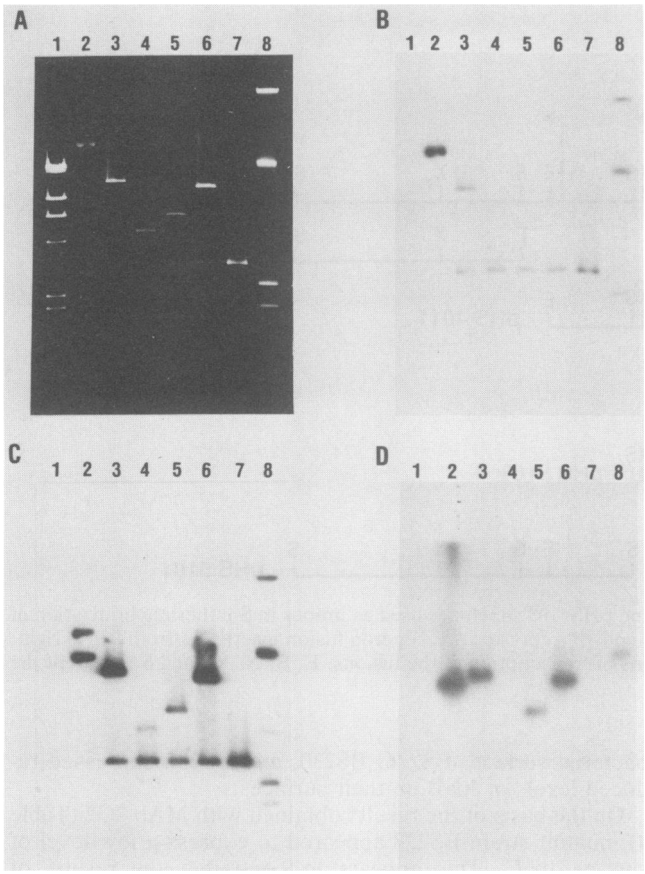

FIG. 3. (A) Ethidium bromide-stained 0.7% agarose gel; (B, C, and D) autoradiograms of Southern hybridization of gel in panel A, using pHS5101 (B), pHS5103 (C), and pHS4011 (D) as probes. Lanes: 1, λ *Hin*dIII size markers; 2, probe DNAs; 3, *Eco*RI-digested pAEH006 (fusion 11.5); 4, *Eco*RI-digested pAEH007 (fusion 17.6); 5, *Eco*RI-digested pAEH008 (fusion 17.7); 6, *Eco*RI-digested pAEH009 (fusion 18.15); 7, *Eco*RI-digested pMLB524; 8, undigested 2457T plasmid DNA.

temperature-regulated virulence phenotype (15). Genes coding for different virulence factors have been identified and cloned, yet the total number of virulence genes and the number of virulence genes which are temperature regulated are not known. We have identified seven temperature-regulated operon fusions in *S. flexneri* 2a by using the λp*lac*Mu *lacZ* operon fusion system (5), expanding on a previous study by Maurelli and Curtiss (17) which first employed this approach. We examined what roles the genes marked by the fusions may play in the virulence phenotype of the organism and determined that these seven insertional mutations identified plasmid-encoded, temperature-regulated genes which could be categorized into three distinct classes: (1) virulence genes involved in the invasion step which are regulated by *virR*, (2) non-virulence-associated genes which are also under the control of *virR*, and (3) non-virulence-associated genes which are independent of *virR* control.

Four of the temperature-regulated fusion mutants isolated were characterized as harboring *lacZ* operon fusions to genes which were involved in expression of the Inv^+ phenotype, as they were unable to invade HeLa cells. In addition, assay of these fusion mutants for the virulence-associated phenotypes of Congo red binding and contact hemolysis, indicated a strong correlation between the Inv phenotype and Pcr^- and Hly^- phenotypes. These data suggest that the genes inactivated by the operon fusions in the Pcr^- mutants, BS226, BS230, and BS232, are also involved in the expression of the Congo red-binding phenotype. On the basis of these results and the ELISA data, discussed below, it appears that the genes inactivated by these fusions, in addition to coding for proteins involved in the invasion step of *Shigella* spp. virulence, may code for proteins involved in the surface expression of the Congo red-binding factor(s), as well as factors involved in contact-mediated hemolytic activity.

In contrast to the Inv^- fusion mutants isolated, three of the seven temperature-regulated *lacZ* operon fusion mutants were still invasive for HeLa cells. Further characterization of these strains in the plaque, Séreny, Congo red binding, and contact hemolytic activity assays indicated that they expressed phenotypic characteristics identical to those of the parental wild-type strain 2457T. These results led us to believe that the genes inactivated by the fusions, although regulated by growth temperature, do not code for products involved in eliciting the virulence phenotype. One must consider, however, the possibility that within the scope of the assay systems used, a mutation in another virulence factor(s) may be undetectable. Another possible explanation for the full-virulence phenotype of these operon fusion mutants is that the gene product resulting from the *lacZ* insertion is not altered sufficiently to be inactivated. This would account for the full-virulence phenotype observed in these strains and the temperature-regulated expression of β-galactosidase. Therefore, these genes may still fall into the class of *virR*-regulated virulence genes (class 1), but further characterization is required in order to confirm this possibility.

Although the 220-kb invasion plasmid presents a much smaller target, relative to the chromosome, for random insertion of the fusion phage, all three classes of temperature-regulated operon fusions were located on the 220-kb virulence plasmid. Detailed mapping of our class 1 mutants placed the fusion end joints on the 37-kb region of the invasion plasmid, defined as the minimum sequence required to confer an Inv^+ phenotype on a plasmidless *Shigella* strain (14). Possible explanations for the absence of chromosomally located virulence gene fusions could be that virulence genes on the chromosome are (i) not temperature regulated and therefore would not be detected by the screening method we employed, (ii) essential genes into which insertions would result in lethal events, or (iii) not detectable due to a screening bias in the virulence assay systems used. In contrast, the data reported here suggest that (i) the genes which contribute to expression of the Inv^+, Pcr^+, and Hly^+ phenotypes are the only virulence genes regulated by temperature and (ii) pHS4108 defines the temperature-regulated virulence regulon in *Shigella* spp.

The fusion in BS228, *ipaB*::*lacZ*$^+$ 17.6, resulted in the synthesis of a truncated IpaB protein and caused a polar mutation which prevented expression of the other *ipa* genes. The nucleotide sequences of *ipaB* and *ipaC* have been determined and show that *ipaB* is the first gene in the operon (1, 29). The polar effect of the *ipaB*::*lacZ*$^+$ 17.6 mutation confirms the operon nature of these genes previously reported. The avirulence of BS228 confirms the involvement of the *ipa* genes in invasion but cannot implicate a specific gene.

Western blot analysis of fusion mutants BS226, BS230, and BS232 demonstrated that insertional mutations in these

strains had no effect on intracellular expression of the Ipa proteins. However, a whole-cell ELISA, used to detect surface expression of the IpaB and IpaC proteins in the mutant strains, indicated an altered recognition of both proteins by the respective MAbs. These mutations could therefore be defects in genes involved in (i) posttranslational modification of the Ipa proteins or (ii) transport and positioning of the Ipa proteins in the outer membrane of the bacterium. Another interpretation of the ELISA data is that the Ipa proteins are surface expressed but masked in such a fashion that they are not recognized by the MAbs. However, preliminary analysis of membrane fractions indicates that the Ipa proteins are not surface expressed in the mutant strains. On the basis of these observations, we believe that the insertional mutations in strains BS226, BS230, and BS232 are in genes essential for membrane expression of the Ipa proteins (*mxi* genes).

Six of the insertional mutations we isolated fell into two distinct classes (class 1 and class 2) with respect to their effect on the virulence phenotype. Although each of the promoters identified by these two classes of fusions were regulated by *virR*, they differed in the degree to which they were regulated. All of the *inv*::*lacZ* fusion mutants expressed a 37-to-30°C β-galactosidase ratio of >50. In contrast, the *lacZ* fusion mutants unaffected in their virulence phenotype expressed a ratio of <10. Moreover, the levels of β-galactosidase for the *inv*::*lacZ* fusion mutants at 37°C were higher (>300 U) and the levels at 30°C were lower (<10 U) than the levels expressed in the Inv^+ temperature-regulated *lacZ* operon fusion mutants (≤340 U at 37°C and 40 to 55 U at 30°C). Thus, the *inv*::*lacZ* fusion mutants expressed a greater degree of regulation in response to temperature change, with a higher level of transcription of these genes at 37°C and an absence of transcription at 30°C. These observations indicate that the temperature-dependent coordinate regulation of virulence genes in *Shigella* spp. may function in such a way as to allow the bacteria to turn virulence genes on to a high level or completely off, as needed. Bacteria in an environment outside the host (<30°C) would be at a selective advantage in being able to conserve energy by not synthesizing virulence gene products, while inside the host the same bacteria would have a selective advantage by being able to turn on virulence genes and thus invade host epithelial tissue. The temperature regulation of β-galactosidase in the Inv^+ mutants suggests, however, that virulence genes are not the only genes regulated by temperature. The data indicate that a more subtle regulation may be employed by these nonvirulence genes in response to a temperature shift from 37 to 30°C. These genes appear to be actively transcribed at both temperatures, yet the difference in the levels of expression between the two temperatures is less pronounced than for the *inv*::*lacZ* genes. It is clear, however, that the results reported here confirm and extend previous observations (17) that temperature-dependent expression of virulence genes in *Shigella* spp. is regulated by *virR*.

The possible existence of another *virR*-like repressor is suggested by the third class of insertional mutants we isolated, as fusion mutant BS236 showed no change in β-galactosidase expression after the introduction of a *virR*::Tn*10* mutation. Other studies (23; B. Adler, C. Sasakawa, T. Tobe, S. Makino, K. Komatsu, and M. Yoshikawa, Mol. Microbiol., in press) have demonstrated regulatory activity of genes other than *virR* in the expression of *Shigella* virulence, yet these have all been identified as activators. In characterizing one of our *inv*::*lacZ* fusions we too have identified a promoter which requires a virulence plasmid-encoded activator (A. E. Hromockyj and A. T. Maurelli, manuscript in preparation).

The use of *lacZ* operon fusion technology has allowed us to identify three novel temperature-regulated virulence genes in *S. flexneri* 2a and to study the possible function of these genes in the invasion phenotype. Moreover, the isolation of these *lacZ* fusions will allow us to isolate temperature-regulated virulence gene promoters in order to further investigate the molecular mechanisms of temperature-dependent regulation of *Shigella* spp. virulence.

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