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Bacteriophage Mu *d*1(Ap^r *lac*) Generates *vir-lac* Operon Fusions in *Shigella flexneri* 2a

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Previous studies have demonstrated that expression of virulence in *Shigella* spp. is controlled by growth temperature. To study the regulation of virulence (*vir*) genes, we set out to develop a rapid, easily-assayed phenotype with which to measure expression of virulence. This report described a procedure for isolating *vir-lac* operon fusions in *S. flexneri* 2a by using the specialized transducing bacteriophage Mu *d*1(Ap^r *lac*) of Casadaban and Cohen (M. Casadaban and S. N. Cohen, Proc. Natl. Acad. Sci. U.S.A. 76:4530–4533, 1976). Mu *d*1(Ap^r *lac*) lysogens were isolated and screened for loss of virulence and for temperature-dependent expression of the lactose genes on Mu *d*1(Ap^r *lac*). A recombinant plasmid carrying the Mu immunity gene was also introduced into lysogens of interest to stabilize the Mu *d*1(Ap^r *lac*) insertion and prevent possible thermal induction at 37°C. The mutant which we isolated failed to penetrate tissue culture cells in the assay for virulence and produced almost 15-fold more β-galactosidase when grown at 37°C than when grown at 30°C. The site of insertion of Mu *d*1(Ap^r *lac*) in this strain was shown to be in the 140-megadalton plasmid pSf2a140, which is known to be associated with virulence. P1L4-mediated transduction of the insertion into a virulent recipient demonstrated genetic linkage of Mu *d*1(Ap^r *lac*) with loss of virulence and temperature-dependent expression of β-galactosidase. All of these features fulfill the phenotype expected for a Mu *d*1(Ap^r *lac*)-induced *vir-lac* operon fusion. This mutant provides us with a means of measuring expression of a gene function required for virulence by assaying for β-galactosidase. The insertion will also serve as a starting point for mapping of genes on pSf2a140 which are necessary for expression of virulence.

One of the causative agents of bacillary dysentery in humans is *Shigella flexneri*. An important feature of the pathogenicity of the organism is its ability to penetrate and multiply within the epithelial cells of the large intestine (20). The invasive ability of the organism is responsible for the pathology observed in disease produced by shigellae (13). Analysis of the virulence determinants of shigellae has been aided by the availability of both animal (30) and tissue culture (15) assays for virulence. The early use of these assays allowed the identification of chromosomal regions which appear to be necessary for virulence (11, 12, 14). More recent reports utilizing these assays have demonstrated the association of large plasmids with the virulence of *Shigella sonnei* and *S. flexneri* (28, 29).

Our laboratory has been investigating the genetic and physiological parameters involved in the expression of virulence in *S. flexneri* 2a. We have examined the regulation of expression of virulence genes in *Shigella* spp. and found expression to be dependent on growth temperature (24). However, the difficulty in studying regulation of virulence gene expression lies in the fact that the gene products involved have not been identified, and the assays for virulence, although useful on a small scale, are not easily adapted to scaling up for screening thousands of potential mutants. These problems are not unique to the genetic analysis of virulence determinants, and genetic solutions to analogous problems in other bacterial systems have been developed over the years. In particular, the operon fusion technique of Casadaban and Cohen (4) has been widely used to obtain lactose (*lac*) operon fusions for studying the regulation of expression of a variety of operons in *Escherichia coli* (for examples, see references 1 and 26) and *Salmonella typhimurium* (6). This technique utilizes a derivative of bacteriophage Mu called Mu *d*1(Ap^r *lac*) and is particularly suitable for studying the regulation of genes whose products are difficult to assay or are unknown, as is the case for expression of virulence genes in *S. flexneri*.

To facilitate our study of the regulation of expression of virulence genes in *S. flexneri* 2a, we decided to isolate fusions of Mu *d*1(Ap^r *lac*) to genes anticipated to be necessary for virulence, that is, *vir-lac* operon fusions. The experiments described in this report were designed to isolate such fusion strains, making use of the fact that expression of virulence in *S. flexneri* 2a is regulated by growth temperature. We therefore looked for Mu *d*1(Ap^r *lac*) insertions which would abolish virulence of the strain and bring expression of the *lac* genes under the control of promoters for these presumptive virulence genes, thus making the strain temperature-dependent for expression of *lac*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. flexneri* 2a mutants described in this paper were derived from strain 2457T, a virulent isolate of *S. flexneri* 2a (10) and are listed in Table 1. This strain carries a 140-megadalton (Mdal) plasmid associated with virulence (29) which we designate as pSf2a140. Strain 2457T also has a large 105-Mdal cryptic plasmid, as well as two small cryptic plasmids with molecular masses of 2.6 and 2.0 Mdal (17). As is common with other isolates of *S. flexneri*, strain 2457T does not ferment lactose and requires nicotinic acid and aspartic acid for optimal growth in minimal salts medium (20).

E. coli MAL103 (4), a lysogen carrying both Mu *d*1(Ap^r *lac*) and a helper Mu *cts*62, was used to produce lysates of Mu *d*1(Ap^r *lac*) as described below.

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TABLE 1. Bacterial strains and plasmid content

Strain	Plasmid content[a]	Derivation of strain (reference)
2457T	pSf2a140	From S. B. Formal (10)
24570	pSf2a140	Spontaneous avirulent derivative of 2457T (10)
BS115	pSf2a140; pGW600	pGW600 transformant of 2457T
BS120	pSf2a140::Mu *d*1(Ap^r *lac*)-83	Mu *d*1(Ap^r *lac*) mutant of 2457T
BS121	pSf2a140::Mu *d*1(Ap^r *lac*)-83; pGW600	pGW600 transformant of BS120
BS125	pSf2a140::Mu *d*1(Ap^r *lac*)-23	Mu *d*1(Ap^r *lac*) mutant of 2457T
BS147	pSf2a140::Mu *d*1(Ap^r *lac*)-83	*galU*::Tn*10* derivative of BS120
BS149	pSf2a140	Mu *d*1(Ap^r *lac*) mutant of 2457T which requires methionine for growth in minimal media
BS150[b]	pSf2a140	Mu *d*1(Ap^r *lac*) mutant of 2457T
BS151	pSf2a140::Mu *d*1(Ap^r *lac*)-23; pGW600	pGW600 transformant of BS125
BS152	pSf2a140::Mu *d*1(Ap^r *lac*)-83; pGW600	Ap^r transductant of BS115 using P1L4 grown on BS147
BS155[c]	pSf2a140	Mu *d*1(Ap^r *lac*) mutant of 2457T

[a] Strain 2457T and all of the derivatives listed also carry three cryptic plasmids with molecular masses of 105, 2.6, and 2.0 Mdal.

[b] BS150 was chosen as a Lac$^+$ control. It is Lac$^+$ at 30 and 37°C, has no new nutritional requirements, and is invasive in the Henle assay.

[c] BS155 was chosen as a Lac$^-$ control and is Lac$^-$ at 30 and 37°C.

Plasmid pGW600 (obtained from G. Walker) consists of the cloning vector pMB9 into which the left terminal *Hin*dIII fragment of Mu has been inserted (18). The Mu repressor encoded by the gene located on this fragment in pGW600 is temperature stable and thus enabled us to use pGW600 to stabilize Mu *d*1(Ap^r *lac*) lysogens at 37°C. Strains carrying pGW600 were grown in the presence of 12.5 μg of tetracycline hydrochloride (Sigma Chemical Co., St. Louis, Mo.) per ml to maintain selective pressure for the plasmid.

Media and growth conditions. L broth (21) was used as complete medium for the growth of bacteria. For work with bacteriophage P1L4, L broth, L agar, and L soft agar (0.65% agar) all contained 5 mM $CaCl_2$. Antibiotic medium no. 2 (Difco Laboratories, Detroit, Mich.) containing 25 μg of ampicillin (Sigma) per ml and 0.8% NaCl was used for selection of Mu *d*1(Ap^r *lac*) lysogens. Scoring for the ability to use lactose was done on minimal salts agar (7) supplemented with 20 μg of nicotinic acid per ml, 20 μg of aspartic acid per ml, and 0.5% lactose. Identification of auxotrophs was also done on minimal salts agar supplemented with aspartic acid and nicotinic acid and containing 0.5% glucose as the carbon source. Strains lysogenized with Mu *d*1(Ap^r *lac*) were always grown at 30°C, except where indicated.

Mutagenesis by bacteriophage Mu *d*1(Ap^r *lac*) and screening for *vir-lac* operon fusions. Lysates of Mu *d*1(Ap^r *lac*) were prepared by heat induction of strain MAL103 as described previously (4).

Log-phase cultures of virulent *S. flexneri* 2a strain 2457T grown at 37°C were mutagenized with Mu *d*1(Ap^r *lac*) as described by Casadaban and Cohen (4). A multiplicity of 0.1 was used to minimize the chance of isolating mutants which were doubly lysogenic for Mu *d*1(Ap^r *lac*). Initial selection of ampicillin-resistant (Ap^r) insertion mutants which were Lac$^+$ (lactose fermenting) at 37°C might have resulted in partial induction of the prophage due to the temperature-sensitive nature of the Mu *d*1(Ap^r *lac*) repressor (4). This could result in genetic rearrangements (9, 33) which would have complicated analysis of the mutants. Therefore, Mu *d*1(Ap^r *lac*)-induced Ap^r insertion mutants were selected at 30°C. Mutants were screened for temperature-regulated expression of the *lac* genes on Mu *d*1(Ap^r *lac*) by using sterile toothpicks to patch colonies onto duplicate plates of minimal salts agar containing 0.5% lactose and onto a minimal plate containing 0.5% glucose as the carbon source. The minimal plate containing glucose served as the master plate and was incubated at 30°C along with one minimal plate containing lactose. The duplicate minimal lactose plate was incubated at 37°C. Strains which were Lac$^+$ at 37°C but Lac$^-$ at 30°C after 24 h were purified from the master plate, grown at 30°C, and subcultured at 37°C to assay for invasiveness in the Henle assay.

Virulence assays. The ability of strains to invade Henle intestinal epithelial cells in tissue culture was used as an assay for determination of virulence. Nonconfluent monolayers of Henle cells were challenged with bacteria as previously described (24). Stained monolayers were examined by phase-contrast microscopy for the presence of bacteria within Henle cells. Invasive shigellae strains generally invaded 90 to 95% of the cells in a challenged monolayer.

The ability of strains to produce keratoconjunctivitis in a guinea pig (Sereny test [30]) was also used as an assay for virulence. Bacteria grown at 37°C were washed once with buffered-saline gelatin (7) and concentrated, and a 25-μl sample containing ca. 10^9 organisms was gently dripped onto the eye of an adult albino guinea pig. Strains which produced keratoconjunctivitis within 3 days were considered virulent.

Transformation of bacteria with plasmid DNA. Purified plasmid pGW600 DNA was introduced into strains of *S. flexneri* 2a by transformation, using the procedure of Gill and Alexander (see reference 8). Transformants were selected on plates containing 12.5 μg of tetracycline per ml.

Generalized transduction with bacteriophage P1L4. The procedures involving P1L4 have been previously described (8) and were modified for use with Mu *d*1(Ap^r *lac*) lysogens of *S. flexneri* 2a. Donor strains in transduction experiments were grown at 30°C to a density of 2×10^8/ml. P1L4 was added to 1.0 ml of cells at a multiplicity of 0.02. Phage was permitted to absorb to the cells for 30 min at 30°C, and then 200 μl of the adsorption mixture was added to 2.5 ml of L soft agar and immediately poured onto a freshly made L agar plate. Two plates were prepared for each lysate. After the agar had solidified, the plates were incubated at 30°C until confluent lysis had occurred, usually after 7 to 8 h. The soft agar was scraped from the plates into a centrifuge tube, the surfaces of the plates were washed with 2 ml of 0.01 M $MgSO_4$, and the wash was added to the centrifuge tube. Chloroform (0.5 ml) was added, and the centrifuge tube was vigorously vortexed for 1 min. Agar and cell debris were pelleted by centrifugation at 10,000 rpm for 10 min at room temperature in a Sorvall SS-34 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The supernatant fluid was titered for PFU and stored at 4°C. P1L4 lysates grown on *S. flexneri* 2a strains yielded 1×10^{10} to 3×10^{10} PFU/ml.

Recipient strains for P1L4 transduction were grown at 37°C to a density of 2×10^8/ml. Phage was added to cells at a multiplicity of 1 to 3 and allowed to adsorb for 20 min at 37°C. Transductants were selected on media containing 0.01 M sodium citrate to prevent readsorption of phage released by lysing cells to surviving transductant cells.

Isolation and characterization of plasmid DNA. The rapid isolation procedure of Kado and Liu (16) was used for routine screening of bacterial strains for altered plasmid profiles. Plasmid DNA used in restriction digests was isolat-

isolated by the procedures of Birnboim and Doly (2) or Casse et al. (5). Digestion of plasmid DNA with restriction endonucleases was performed by the method of Maniatis et al. (23). For DNA-DNA hybridization experiments, plasmid DNA from 0.7% agarose gels was transferred onto nitrocellulose filters by the method of Southern (31) as modified by Maniatis et al. (23). α-^{32}P-labeled Mu DNA was used as a probe to identify Mu *d*1(Ap^r *lac*) sequences, and hybridization of filters was carried out as described by Maniatis et al. (23).

β-Galactosidase assays. β-Galactosidase activity was measured by the method of Miller by using chloroform-sodium dodecyl sulfate treated cells (25). Cultures were grown at 30°C, subcultured into L broth, and grown at the indicated temperature for at least six generations before assaying for β-galactosidase. Enzyme assays were performed at room temperature.

RESULTS

Strategies for mutagenesis. The isolation of *vir-lac* operon fusions makes use of the specialized transducing bacteriophage Mu *d*1(Ap^r *lac*), whose structure is shown in Fig. 1. Like phage Mu itself, Mu *d*1(Ap^r *lac*) is a translocatable element capable of insertion into random sites in bacterial chromosomal and plasmid DNA (3). Mu *d*1(Ap^r *lac*) also contains the genes for expression of ampicillin resistance, which can be used for selection of Mu *d*1(Ap^r *lac*) insertion mutants. Construction of operon fusions is made possible by the presence on the phage of the structural genes of the *lac* operon but without the *lac* promoter. There is no other promoter in the phage from which the *lac* genes may be transcribed. Insertion of Mu *d*1(Ap^r *lac*) in the proper orientation into a transcriptionally active gene *x* results in expression of the *lac* genes due to readthrough transcription from the promoter of gene *x*. Measurement of β-galactosidase activity, a product of the *lacZ* gene, therefore becomes a rapid and simple assay for the activity of the promoter to which the Mu *d*1(Ap^r *lac*) has been fused.

As is true with other translocatable elements, insertion of Mu *d*1(Ap^r *lac*) into gene *x* usually abolishes expression of that gene due to disruption of the continuity of the gene. Therefore, a strategy for isolating Mu *d*1(Ap^r *lac*) operon fusions involves screening Ap^r mutants for the loss of function of gene *x* and then screening these mutants for regulation of expression of *lac* under conditions which regulate expression of gene *x*. Because expression of at least some traits necessary for virulence of *S. flexneri* 2a is regulated by growth temperature (24), we could predict that *vir-lac* operon fusions would result in temperature-dependent expression of the *lac* genes. It was easier for us to score the Lac phenotype, so we first identified mutants which were Lac$^+$ at 37°C and Lac$^-$ at 30°C and then screened these strains for loss of virulence.

Isolation of *vir-lac* operon fusions. Strain 2457T was infected with Mu *d*1(Ap^r *lac*), and Ap^r lysogens were selected at 30°C. Ap^r lysogens arose at a frequency of 3×10^{-3} to 4×10^{-3} per input PFU. When screened for auxotrophs, ca. 2 to 5% of the lysogens were found to have acquired new nutritional requirements. One of these, BS149, was further characterized as being a methionine auxotroph and was used as a control in later experiments. We screened ca. 15,000 Ap^r lysogens for the Lac$^+$ phenotype at 30 and 37°C by patching colonies onto minimal salts plates containing lactose. Between 4 and 5% of the Mu *d*1(Ap^r *lac*) lysogens were Lac$^+$ at 30°C. About 140 of the 15,000 lysogens scored appeared to be Lac$^+$ only at 37°C and not at 30°C. Of these mutants, 107 were assayed for virulence by the Henle cell culture method. Because virulence in *S. flexneri* 2a is not expressed in cultures grown at 30°C, we subcultured the Mu *d*1(Ap^r *lac*) lysogens into L broth and grew them at 37°C for 2 h before assaying invasiveness in Henle cells. We had previously demonstrated that growth at 37°C for 2 h was sufficient to restore expression of virulence in cultures of virulent shigellae which had been growing at 30°C (24). We identified one Mu *d*1(Ap^r *lac*) mutant which was Lac$^+$ at 37°C and Lac$^-$ at 30°C and which failed to invade Henle cells. We called this mutant BS120. The mutants which were Lac$^+$ at 37°C and Lac$^-$ at 30°C but retained the ability to penetrate Henle cells probably represent *lac* operon fusions to other temperature-regulated promoters which control expression of genes unrelated to virulence (see below).

We were aware of the possibility that partial induction of the Mu *d*1(Ap^r *lac*) prophage may occur at 37°C due to the temperature-sensitive nature of the Mu repressor encoded by the phage (4). In *E. coli* Mu cts62 lysogens, partial induction at 37°C results in a 50% survival rate relative to growth of the lysogen at 30°C (9). We found that growth of *S. flexneri* 2a Mu *d*1(Ap^r *lac*) lysogens at 37°C for 2 h in preparation for assaying infectivity in Henle cells did not appreciably affect the growth of the lysogens (data not shown). Moreover, control experiments indicated that either incubation did not occur or occurred at a low frequency and otherwise did not affect the reliability of the assay in determining the invasiveness of a strain. For example, BS149, which we identified as *met*::Mu *d*1(Ap^r *lac*), was still capable of invading both Henle cells and guinea pig conjunctiva after growth at 37°C (Table 2). Therefore, we concluded that the presence of the Mu *d*1(Ap^r *lac*) prophage in a strain does not necessarily interfere with its expression of virulence at 37°C as measured by the Henle assay and the Sereny test.

As an additional precaution against the induction of Mu *d*1(Ap^r *lac*) at 37°C, we transformed several of our mutants with plasmid pGW600. This plasmid carries the gene for the wild-type Mu repressor protein, and bacteria possessing this plasmid produce large amounts of repressor. The presence

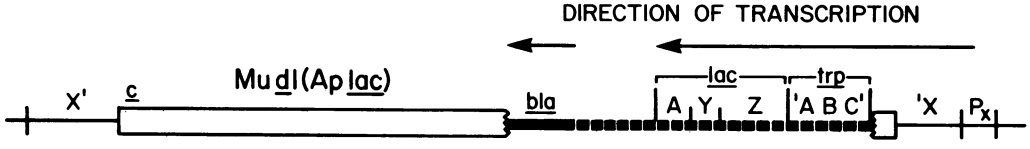

FIG. 1. Schematic representation of the genome of Mu *d*1(Ap^r *lac*) inserted into gene *x* in the orientation which fuses the *lac* genes with the promoter of gene *x* (P_x). The boxed regions denote the bacteriophage Mu *c* and *S* ends which are present in Mu *d*1(Ap^r *lac*). The *bla* gene, represented by the solid box, encodes β-lactamase and confers ampicillin resistance on a Mu *d*1(Ap^r *lac*) lysogen. Transcription of the *bla* gene is from its own promoter. Structural genes, without promoters, from the *lac* and tryptophan (*trp*) operons are shown by dotted lines. The genetic regions shown are not drawn to scale.

TABLE 2. Virulence properties of Mu *d*1(Ap^r *lac*) mutants

Strain	Mu *d*1(Ap^r *lac*) insertion	pGW600	Virulence in assays[a]:	
			Henle	Sereny
2457T	None	−	+	+
2457O	None	−	−	−
BS120	pSf2a140::Mu *d*1(Ap^r *lac*)-83	−	−	ND[b]
BS121	pSf2a140::Mu *d*1(Ap^r *lac*)-83	+	−	−
BS125	pSf2a140::Mu *d*1(Ap^r *lac*)-23	−	+	ND
BS151	pSf2a140::Mu *d*1(Ap^r *lac*)-23	+	+	+
BS149	*met*::Mu *d*1(Ap^r *lac*)-1	−	+	+

[a] Assays for virulence were done with cultures grown at 37°C.
[b] ND, Not determined.

of this plasmid in a Mu *d*1(Ap^r *lac*) lysogen should therefore preclude induction of the bacteriophage at 37°C, since the repressor produced by pGW600 is temperature stable (18). Results of virulence assays of Mu *d*1(Ap^r *lac*) mutants carrying pGW600 were the same as the results seen with mutants without the repressor plasmid (Table 2).

Characterization of plasmid DNA in Mu *d*1(Ap^r *lac*) mutants. It has previously been demonstrated that plasmid pSf2a140 in *S. flexneri* 2a is associated with virulence (29). If a *vir* gene located on pSf2a140 was regulated by growth temperature, we would expect that some of our *vir-lac* operon fusions may be created by insertion of Mu *d*1(Ap^r *lac*) into that gene. Insertion of the 24.4-Mdal genome of Mu *d*1(Ap^r *lac*) into pSf2a140 would increase the size of the plasmid to ca. 165 Mdal, and this increase in size would be detectable on an 0.7% agarose gel. In the course of screening our Mu *d*1(Ap^r *lac*) lysogens, we identified a mutant which appeared to have acquired a large insertion in pSf2a140. This mutant, which we designated BS125, still retained its ability to invade Henle cells, and a pGW600 transformant of this mutant was virulent in the Sereny test (Table 2). BS125 also displayed partial temperature regulation of *lac*, which we will describe in a later section. We suspected that the insertion in pSf2a140 may be due to integration of Mu *d*1(Ap^r *lac*), and therefore we further characterized the plasmid DNA from BS125. Strain BS120, which had the phenotype of a *vir-lac* operon fusion, was also examined for alterations in the molecular makeup of plasmid pSf2a140.

The plasmid DNA profiles of mutants BS120 and BS125 are shown in Fig. 2A, along with the profile of the parent strain, 2457T. Both mutants had lost a plasmid band of ca. 140 Mdal and acquired a new band which migrated more slowly on an 0.7% agarose gel. The electrophoretic mobility of these new bands indicated a molecular mass greater than 140 Mdal, which was consistent with a Mu *d*1(Ap^r *lac*) insertion in pSf2a140. To verify that Mu *d*1(Ap^r *lac*) had indeed inserted into pSf2a140 in the mutants, we transferred the plasmid DNA from the gel shown in Fig. 2A onto a nitrocellulose filter and hybridized the filter with labeled Mu DNA to identify Mu-specific sequences. The new large plasmid bands present in BS120 and BS125 hybridized with the Mu probe, whereas plasmid DNA from the parent strain 2457T did not show any homology with the Mu probe (Fig. 2B). We therefore concluded that Mu *d*1(Ap^r *lac*) had inserted into pSf2a140 in both mutant strains.

An insertion of Mu *d*1(Ap^r *lac*) in pSf2a140 should cause the disappearance of a restriction fragment and the appearance of new fragments whose total sizes should be equal to the size of the lost fragment plus the size of the inserted DNA. Based on a knowledge of the location of restriction sites within Mu *d*1(Ap^r *lac*), the sizes of the new fragments can be predicted. *Eco*RI cuts Mu *d*1(Ap^r *lac*) at three sites, generating internal fragments of 7.65 and 19.7 kilobases (kb) and end fragments of 5.2 and 4.6 kb (27). An *Eco*RI digest of pSf2a140 containing a Mu *d*1(Ap^r *lac*) insertion would therefore generate four new fragments not present in the *Eco*RI digestion pattern of pSf2a140. The 7.65- and 19.7-kb fragments should be found in the *Eco*RI digestion patterns of all Mu *d*1(Ap^r *lac*) mutants regardless of the site of insertion in pSf2a140, since these fragments originate from the interior of the Mu *d*1(Ap^r *lac*) genome. However, the left- and right-end fragments, being fused to neighboring host sequences, should generate different-sized fragments depending on the site of insertion. The sizes of fragments generated would depend on the location of the nearest *Eco*RI site downstream from the insertion. Thus, the size of the left-end fragment would be 5.2 kb + X, and the right-end fragment would be 4.6 kb + Y; $X + Y$ = the size of the restriction fragment into which the insertion has occurred. Insertions of Mu *d*1(Ap^r *lac*) at different sites should yield new end fragments of different sizes, i.e., 5.2 kb + X' and 4.6 kb + Y'.

Although both BS120 and BS125 had Mu *d*1(Ap^r *lac*) insertions on pSf2a140, only BS120 had become avirulent. If the insertion of Mu *d*1(Ap^r *lac*) in BS120 was in a gene necessary for expression of virulence, we expected the restriction digestion pattern of plasmids from this strain to differ from the digestion pattern from BS125, which had a Mu *d*1(Ap^r *lac*) insertion which did not alter virulence. Moreover, the digestion patterns of both mutants should differ from the parent strain 2457T because of the Mu *d*1(Ap^r *lac*) insertion.

Total plasmid DNA from mutants BS120 and BS125 and their parent strain, 2457T, was digested with *Eco*RI or *Sal*I

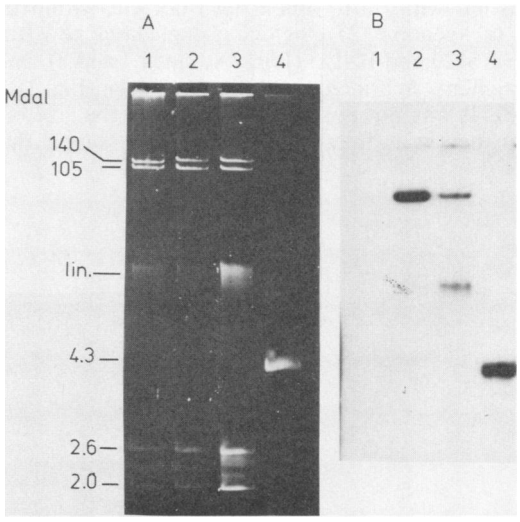

FIG. 2. Analysis of plasmid DNA from 2457T and Mu *d*1(Ap^r *lac*) mutants. Plasmid DNA was fractionated by electrophoresis through an 0.7% vertical agarose gel in Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8]) at 100 V for 6 h. (A) Ethidium bromide-stained 0.7% agarose gel containing plasmid DNA from 2457T (lane 1), BS120 (lane 2), BS125 (lane 3), and plasmid pGW600 (lane 4). (B) Autoradiograph of the Southern transfer of the gel in (A) to which ^{32}P-labeled bacteriophage Mu DNA has hybridized. Plasmid pGW600, which contains the left terminal *Hin*dIII fragment of bacteriophage Mu, served as the positive control for the hybridization. lin, linear DNA.

and analyzed on 0.7% agarose gels. When compared with the profile of 2457T, BS120 was missing an *Eco*RI fragment of 12.5 kb, whereas BS125 lacked an *Eco*RI fragment of 22 kb (Fig. 3A, lanes 2, 3, and 4). This indicated that the site of Mu *d*1(Ap^r *lac*) insertion was different in each mutant. The insertion of Mu *d*1(Ap^r *lac*) into the 12.5-kb *Eco*RI fragment in BS120 generated two new fragments of 9.4 and 12.9 kb which represented the end fragments of Mu *d*1(Ap^r *lac*) fused to adjacent plasmid sequences. As expected, the sum of the sizes of the new fragments (9.4 + 12.9 kb) was equal to the sum of the ends of Mu *d*1(Ap^r *lac*) (4.6 + 5.2 kb) plus 12.5 kb, the size of the fragment into which Mu *d*1(Ap^r *lac*) had inserted. Blot transfer and hybridization of the restricted DNA (Fig. 3A) with ^{32}P-labeled Mu DNA verified the presence of Mu sequences in the 9.4-kb fragment (Fig. 3B, lane 3). The probe did not hybridize well with the 12.9-kb fragment because it contained plasmid DNA fused to the right-end fragment of Mu *d*1(Ap^r *lac*), which contains mostly *E. coli lac* and *trp* sequences and only a very small piece of Mu at the end (Fig. 1 [27]).

Insertion of Mu *d*1(Ap^r *lac*) into the 22-kb *Eco*RI fragment of BS125 resulted in a new fragment of 4.9 kb (Fig. 3A, lane 4). This fragment did not hybridize well with the Mu probe for the same reason outlined above. However, the hybridization did reveal the location of the second predicted new fragment, which was not distinguished on the gel because of the presence of other fragments of plasmid DNA of similar size (Fig. 3A and B, lane 4). This fragment, which contained the left end of Mu *d*1(Ap^r *lac*), was ca. 26 kb, in close agreement with the predicted size.

In Fig. 3A, the presence of unique fragments derived from within Mu *d*1(Ap^r *lac*) in BS120 and BS125 could not be clearly seen. Nevertheless, hybridization of the gel with ^{32}P-labeled Mu DNA demonstrated their presence and indicated that these fragments were masked by plasmid-derived fragments of similar size (Fig. 3B).

Digestion with *Sal*I, which does not cut within the Mu *d*1(Ap^r *lac*) genome (27), yielded a single large new fragment in both BS120 and BS125 (Fig. 3A, lanes 7 and 8), and both new fragments hybridized with the Mu probe (Fig. 3B, lanes 7 and 8). It was not possible to determine the size of these new fragments, as they were considerably larger than the largest molecular-weight standard on the gel. However, it was evident from the difference in migration that Mu *d*1(Ap^r *lac*) had inserted into a different *Sal*I fragment in each mutant.

Genetic evidence of linkage of Mu *d*1(Ap^r *lac*) insertion with loss of virulence. The presence of Mu *d*1(Ap^r *lac*) on pSf2a140 in the avirulent mutant BS120 was strong evidence that the insertion had inactivated a gene required for virulence. To confirm this, we transduced the Mu *d*1(Ap^r *lac*) insertion into a virulent recipient strain and assayed Ap^r transductants for virulence. P1L4 was grown on BS147 as described earlier. BS147 is a *galU*::Tn*10* derivative of BS120 which we constructed for this purpose since *galU* mutants of *S. flexneri* 2a produce higher-titer P1L4 lysates than do Gal$^+$ strains (unpublished data).

To reduce the possibility of zygotic induction, which might occur upon transduction of Mu *d*1(Ap^r *lac*) into a new host, we used a pGW600 transformant of 2457T, designated BS115, as the recipient. The temperature-stable Mu repressor produced by pGW600 should preclude zygotic induction and also allow transductions to be done at 37°C (18).

BS115 was infected with P1L4 grown on BS147, and selection was made for Ap^r to select for transduction of the Mu *d*1(Ap^r *lac*) insertion. Five Ap^r transductants were purified and tested for invasiveness in the Henle assay. All five transductants failed to penetrate Henle cells, whereas the parent strain, BS115, invaded 90% of the cells in a monolayer. In addition, the transductants displayed temperature-dependent expression of *lac*, being Lac$^+$ at 37°C but Lac$^-$ at 30°C. Examination of the plasmid content of the transductants revealed the absence of a 140-Mdal band and the appearance of a new band migrating in a way similar to that of the slow band of the donor strain, BS147. *Bam*HI digestion of plasmid DNA from one of these transductants, BS152, produced a pattern similar to that seen in *Bam*HI digestion of plasmid DNA from the donor (data not shown). These results are consistent with recombination of a transducing fragment containing the Mu *d*1(Ap^r *lac*) insertion from BS147 into pSf2a140 in the recipient strain. We therefore established genetic linkage of the Mu *d*1(Ap^r *lac*) insertion in BS120 with loss of virulence in that strain.

Assay for β-galactosidase. Preliminary screening of Mu

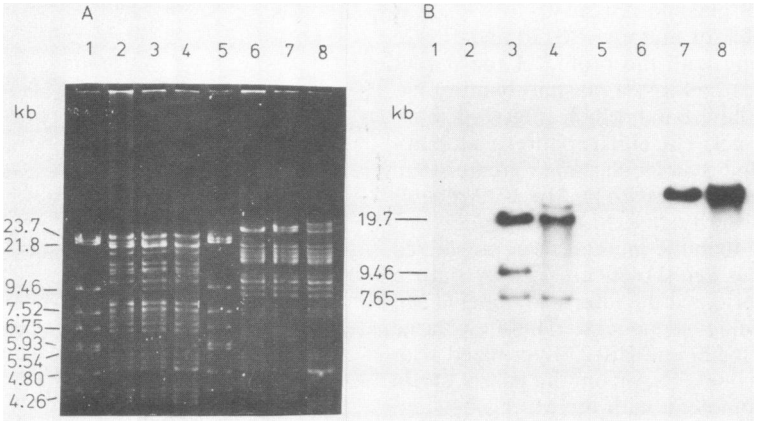

FIG. 3. Restriction endonuclease digestion of plasmid DNA from 2457T and Mu *d*1(Ap^r *lac*) mutants. Electrophoresis was through an 0.7% agarose gel in Tris-borate buffer at 45 V for 22 h. Shown are an ethidium bromide-stained agarose gel (A) and an autoradiogram of Southern blot (B) containing 2457T plasmid DNA digested with *Eco*RI (lane 2) and *Sal*I (lane 6), BS120 plasmid DNA digested with *Eco*RI (lane 3) and *Sal*I (lane 7), and BS125 plasmid DNA digested with *Eco*RI (lane 4) and *Sal*I (lane 8). Lanes 1 and 5 contain mixtures of *Eco*RI and *Hin*dIII digested λ DNA. Hybridization was carried out with ^{32}P-labeled bacteriophage Mu DNA.

*d*1(Ap^r *lac*) mutants had identified BS120 as a mutant which expressed the Lac$^+$ phenotype at 37°C but not at 30°C and was not invasive. To quantitate more precisely the expression of the *lac* operon in BS120, we measured β-galactosidase activity in this strain and in several other Mu *d*1(Ap^r *lac*) mutants as well. The data in Table 3 show the levels of β-galactosidase in strains grown at 30 and 37°C. Since the enzyme assays were done at room temperature for all strains tested, the amount of β-galactosidase activity represents actual enzyme levels produced by the strain and not enzyme activity as affected by assay temperature. BS120 clearly displayed temperature-dependent expression of β-galactosidase, producing ca. 15-fold more enzyme when grown at 37°C than when grown at 30°C. To ensure that the increased β-galactosidase production at 37°C was not due to induction of the Mu *d*1(Ap^r *lac*) prophage at this temperature, we measured the levels of β-galactosidase in BS121, a pGW600 transformant of BS120. This strain also showed temperature-dependent expression of β-galactosidase, similar to that seen in BS120. β-Galactosidase levels in the transductant, BS152, were also significantly increased at 37°C.

Mutant BS125 produced a partial but reproducible increase in β-galactosidase activity at 37°C. Although we have not further characterized the nature of the *lac* fusion in BS125, the higher β-galactosidase level at 37°C does not appear to be due to induction of the prophage at this temperature. BS151, a pGW600 transformant of BS125 which produces stable Mu repressor and blocks prophage induction, also showed an elevated level of β-galactosidase at 37°C compared with the level at 30°C.

The induction of β-galactosidase synthesis due to a shift in growth temperature to 37°C is a special feature of the Mu *d*1(Ap^r *lac*) insertion in BS120 and its derivatives. Strain BS150, which is a Mu *d*1(Ap^r *lac*) lysogen selected for its Lac$^+$ phenotype at 30 and 37°C, produced a constant amount of β-galactosidase at both temperatures. BS155, which expressed barely detectable levels of β-galactosidase at 30°C, did not express very much enzyme activity when grown at 37°C. We therefore concluded that the Mu *d*1(Ap^r *lac*) insertion in BS120 fused the *lac* genes with a promoter which is regulated by growth temperature.

TABLE 3. β-Galactosidase levels in Mu *d*1(Ap^r *lac*) mutants grown at 30 and 37°C

Strain	Mu *d*1(Ap^r *lac*) insertion	Units of β-galactosidase[a] at:		Ratio (37°C/30°C)
		30°C	37°C	
2457T	None	<1	<1	
BS115	None	<1	<1	
BS120	pSf2a140::Mu *d*1(Ap^r *lac*)-83	8.9	128.2	14.4
BS121	pSf2a140::Mu *d*1(Ap^r *lac*)-83	14.0	218.2	15.6
BS125	pSf2a140::Mu *d*1(Ap^r *lac*)-23	24.3	78.8	3.2
BS151	pSf2a140::Mu *d*1(Ap^r *lac*)-23	30.9	276.7	8.9
BS150[b]	Chromosomal Mu *d*1(Ap^r *lac*) insertion 54-9	254.6	244.2	
BS152	pSf2a140::Mu *d*1(Ap^r *lac*)-83	19.3	292.1	15.0
BS155[b]	Chromosomal Mu *d*1(Ap^r *lac*) insertion 55-1	1.8	2.8	1.6

[a] Determination of β-galactosidase specific activity was done at room temperature. Units are those described by Miller (25).

[b] BS150 and BS155 were used as Lac$^+$ and Lac$^-$ controls, respectively. These mutants have no new nutritional requirements and are invasive in the Henle assay.

DISCUSSION

The purpose of this investigation was to develop and utilize a protocol designed to isolate *vir-lac* operon fusions in *S. flexneri* 2a. The particular properties of the organism and the nature of the genetic phenomenon which we wished to study made us choose the fusion technique of Casadaban and Cohen, using Mu *d*1(Ap^r *lac*) (4). *S. flexneri* 2a strains are naturally sensitive to infection by phage Mu, and the organism is also Lac$^-$ and ampicillin sensitive. Thus, there was no need to modify the organism to be able to use the technique. Because the expression of virulence in *S. flexneri* is temperature dependent, we were able to look for the temperature-dependent expression of the *lac* genes of Mu *d*1(Ap^r *lac*) as a preliminary screening for *vir-lac* operon fusions.

Genetic, biochemical, and physical approaches were used to confirm *vir-lac* operon fusions. BS120, the *vir-lac* operon fusion mutant which we described in this report, was identified by the predicted properties that such a fusion mutant should have. The strain was avirulent, and it expressed high levels of β-galactosidase at 37°C, whereas synthesis of the enzyme was repressed at 30°C. P1L4 transduction of ampicillin resistance from BS120 into a virulent recipient was linked with loss of virulence and also linked with temperature-dependent regulation of the *lac* genes of Mu *d*1(Ap^r *lac*). These results provided genetic and biochemical evidence that BS120 contained a *vir-lac* fusion.

The plasmid profile of BS120 was consistent with a Mu *d*1(Ap^r *lac*) insertion in pSf2a140. DNA-DNA hybridization with labeled Mu DNA and analysis of restriction digestion patterns of the plasmids from BS120 further supported this conclusion and indicated that Mu *d*1(Ap^r *lac*) had inserted into a 12.5-kb *Eco*RI fragment.

We isolated a number of mutants which appeared to be Lac$^+$ at 37°C but Lac$^-$ at 30°C, but only BS120 was avirulent. Although quantitative measurements of β-galactosidase levels in all of these mutants were not made, it is likely that we isolated a number of Mu *d*1(Ap^r *lac*) insertions which displayed temperature-dependent expression of *lac*, and yet the fusions did not abolish virulence. We interpret this to mean that there are many other genes in *S. flexneri* 2a which are not related to virulence but which, like the virulence genes, are regulated by growth temperature. Examples of such genes have been identified in other systems. The F-pilin of *E. coli* is synthesized at 37°C but not at 32°C (32), and a recent report described the temperature regulation of the *ompC* and *ompF* gene products, which are outer membrane proteins of *E. coli* (22). In this regard, the partial temperature induction of β-galactosidase in BS125 was interesting. Since the Mu *d*1(Ap^r *lac*) insertion in BS125 did not abolish virulence, the insertion would seem to be outside DNA sequences required for expression of virulence. The insertion may in fact be just inside the promoter distal end of a *vir* gene sequence, perhaps causing no loss of function of the *vir* gene product while allowing limited readthrough transcription into the *lac* genes. Alternatively, the Mu *d*1(Ap^r *lac*) insertion may be fused to a promoter which is regulated by temperature but unrelated to virulence. Further analysis of the location of the Mu *d*1(Ap^r *lac*) insertion in BS125 should establish its relation, if any, to *vir* gene sequences.

The usefulness of having Mu *d*1(Ap^r *lac*) operon fusions has been demonstrated by the many novel and interesting questions which have been answered by using such fusions in other systems. The *vir-lac* operon fusion in BS120 allows us to monitor the expression of virulence genes by measur-

ing the levels of β-galactosidase produced by the strain. Using the production of β-galactosidase as an assay, we have begun searching for regulatory mutants which alter the levels of expression of β-galactosidase in BS120. The mutations in these strains should also alter the expression of virulence genes, and this can be tested by moving the mutation into a virulent strain. Thus, we have developed a powerful tool for examining the regulation of virulence gene expression in *S. flexneri* 2a.

Mapping of virulence genes on pSf2a140 should also be facilitated by the mutants described in this paper. The Mu *d*1(Ap^r *lac*) insertion in BS120 can be used as a starting point for mapping the virulence genes, as this insertion appears to be in a region responsible for virulence. By using labeled junction fragments from the Mu *d*1(Ap^r *lac*) insertion in BS120 to screen a clone bank consisting of plasmid fragments from the virulent parent strain 2457T, we should be able to identify neighboring *vir* gene sequences. The clones thus identified can also be tested for their ability to complement the defects in noninvasive, deletion mutants of pSf2a140. Such a combination of physical and genetic techniques will help us in understanding the organization and regulation of virulence genes on pSf2a140.

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LITERATURE CITED

1. **Bankaitis, V. A., and P. J. Bassford, Jr.** 1982. Regulation of adenylate cyclase synthesis in *Escherichia coli*: studies with *cya-lac* operon and protein fusion strains. J. Bacteriol. **151**:1346–1357.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Bukhari, A. I.** 1976. Bacteriophage Mu as a transposition element. Annu. Rev. Genet. **10**:389–412.
4. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-lac bacteriophage: in vivo probe for transcriptional control sequences. Proc. Natl. Acad. Sci. U.S.A. **76**:4530–4533.
5. **Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Denarie.** 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. **113**:229–242.
6. **Csonka, L. N., M. M. Howe, J. L. Ingraham, L. S. Pierson III, and C. L. Turnbough, Jr.** 1981. Infection of *Salmonella typhimurium* with coliphage Mu *d*1(Ap^r *lac*): construction of *pyr*::*lac* gene fusions. J. Bacteriol. **145**:299–305.
7. **Curtiss, R., III.** 1965. Chromosomal aberrations associated with mutations to bacteriophage resistance in *Escherichia coli*. J. Bacteriol. **89**:28–40.
8. **Curtiss, R., III.** 1981. Gene transfer, p. 243–265. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
9. **Faelen, M., A. Toussaint, and J. De Lafonteyne.** 1975. Model for the enhancement of λ-*gal* integration into partially induced Mu-1 lysogens. J. Bacteriol. **121**:873–882.
10. **Formal, S. B., G. J. Dammin, E. H. LaBrec, and H. Schneider.** 1958. Experimental shigella infections: characteristics of a fatal infection produced in guinea pigs. J. Bacteriol. **75**:604–610.
11. **Formal, S. B., P. Gemski, Jr., L. S. Baron, and E. H. LaBrec.** 1971. A chromosomal locus which controls the ability of *Shigella flexneri* to evoke keratoconjunctivitis. Infect. Immun. **3**:73–79.
12. **Formal, S. B., E. H. LaBrec, T. H. Kent, and S. Falkow.** 1965. Abortive intestinal infection with an *Escherichia coli-Shigella flexneri* hybrid strain. J. Bacteriol. **89**:1374–1382.
13. **Gemski, P., Jr., 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.
14. **Gemski, P., Jr., D. G. Sheahan, O. Washington, and S. B. Formal.** 1972. Virulence of *Shigella flexneri* hybrids expressing *Escherichia coli* somatic antigens. Infect. Immun. **6**:104–111.
15. **Hale, T. L., and P. F. Bonventre.** 1979. Shigella infection of Henle intestinal epithelial cells: role of the bacterium. Infect. Immun **24**:879–886.
16. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
17. **Kopecko, D. J., J. Holcombe, and S. B. Formal.** 1979. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri*. Infect. Immun. **24**:580–582.
18. **Krueger, J. H., and G. C. Walker.** 1983. Mu *d*I(Ap^r *lac*)-generated fusions in studies of gene expression. Methods Enzymol. **100**:501–509.
19. **Kwoh, D., and D. Zipser.** 1979. Specific binding of Mu repressor to DNA. Nature (London) **277**:489–491.
20. **LaBrec, E. H., H. Schneider, T. J. Magnani, and S. B. Formal.** 1964. Epithelial cell penetration as an essential step in the pathogenesis of bacillary dysentery. J. Bacteriol. **88**:1503–1518.
21. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190–206.
22. **Lundrigan, M. D., and C. F. Earhart.** 1984. Gene *envY* of *Escherichia coli* K-12 affects thermoregulation of major porin expression. J. Bacteriol. **157**:262–268.
23. **Maniatis, R., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Maurelli, A. T., B. Blackmon, and R. Curtiss III.** 1984. Temperature-dependent expression of virulence genes in *Shigella* species. Infect. Immun. **43**:195–201.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Mulligan, J. T., W. Margolin, J. H. Krueger, and G. C. Walker.** 1982. Mutations affecting regulation of methionine biosynthetic genes isolated by use of *met-lac* fusions. J. Bacteriol. **151**:609–619.
27. **O'Connor, M. B., and M. H. Malamy.** 1983. A new insertion sequence, IS*121*, is found on the Mu dI1(Ap *lac*) bacteriophage and the *Escherichia coli* K-12 chromosome. J. Bacteriol. **156**:669–679.
28. **Sansonetti, P. J., D. J. Kopecko, and S. B. Formal.** 1981. *Shigella sonnei* plasmids: evidence that a large plasmid is necessary for virulence. Infect. Immun. **34**:75–83.
29. **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.
30. **Sereny, B.** 1955. Experimental *Shigella* conjunctivitis. Acta Microbiol. Acad. Sci. Hung. **2**:293–296.
31. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
32. **Sowa, B. A., D. Moore, and K. Ippen-Ihler.** 1983. Physiology of F-pilin synthesis and utilization. J. Bacteriol. **153**:962–968.
33. **Toussaint, A., M. Faelen, and A. I. Bukhari.** 1977. Mu-mediated illegitimate recombination as an integral part of the Mu life cycle, p. 275–285. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.