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A Chromosomal Locus Which Controls the Ability of *Shigella flexneri* to Evoke Keratoconjunctivitis

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The primary step in the pathogenesis of bacillary dysentery is the penetration of intestinal epithelial cells by shigellae. Lacking this capacity, *Shigella flexneri* becomes avirulent. By means of intergeneric conjugation between various *Escherichia coli* K-12 Hfr strains and *S. flexneri* 2a virulent recipients and by reciprocal transduction analysis with phage P1 *vir*, we established a locus on the genome of *S. flexneri* 2a which is necessary for the ability of this strain to penetrate epithelial cells as measured by the Sereney test for keratoconjunctivitis. This locus, termed *kcpA* (in reference to its involvement in provoking keratoconjunctivitis), has been positioned between the *lac* and *gal* chromosomal markers and is cotransducible with the *purE* allele.

We previously demonstrated that an essential step in the pathogenesis of bacillary dysentery is the penetration of host intestinal epithelial cells by the pathogen (10). Mutant strains of dysentery bacilli which lack the ability to penetrate intestinal epithelial cells are avirulent, and animals fed such cultures react for the most part similarly to those fed *Escherichia coli* (10; H. Schneider and S. B. Formal, Bacteriol. Proc., p. 66, 1963).

Since *Shigella flexneri* is genetically related to *E. coli* (4, 7, 8), it should be possible to prepare shigella hybrids which are unable to penetrate the intestinal barrier. In previous studies of this type, we have already incorporated into the shigella genome segments covering approximately 50% of the *E. coli* chromosome. Such hybrids remained consistently unaltered in their virulence with but one exception; after the *E. coli xyl-rha* region was transferred to shigella, the hybrid strain failed to cause overt disease when fed to experimental animals (4). This hybrid class, however, still retained the ability to penetrate the intestinal barrier. The failure of these hybrids to produce disease was due to an inability of hybrid cells to persist in the intestinal mucosa for a sufficient time after penetration (8).

The purpose of this communication is to summarize recent genetic studies on *S. flexneri* in which we have attempted to alter the ability of the pathogen to penetrate epithelial cells. There are several procedures which one can employ to assess the ability of a shigella strain to penetrate intestinal epithelial cells. The most convenient, we have found, is the Sereney test which measures the capability of the organism to cause keratoconjunctivitis (15). This in turn is a measure of its capacity to penetrate the epithelial cells of the cornea (10, 14). By means of the Sereney test as a screening procedure for epithelial cell penetrability and genetic experiments involving intergeneric matings and transduction, we detected a locus in one area of the chromosome which controls epithelial cell penetration. Since other loci controlling epithelial cell penetration must exist, we have tentatively termed this locus *kcpA* (keratoconjunctivitis provocation).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains employed in this study and their characteristics are listed in Table 1.

Media. Penassay Broth (Difco) and meat extract-agar were employed for routine cultivation of bacterial strains. The composition of minimal medium, used in genetic experiments for selecting and scoring recombinant hybrids, was previously described (4). When required, amino acids and growth factors were added to minimal medium at a final concentration of 25 μg/ml. Streptomycin, employed as a counter-selective agent against male cells in some of the matings, was added to minimal medium at a final concentration of 500 μg/ml. Fermentation characters were scored on MacConkey indicator medium, which consisted of MacConkey Agar Base (Difco) supplemented with appropriate carbohydrate (1%).

Strains employed in transduction experiments and as hosts for phage P1 *vir* were usually cultured in L broth (11). L agar and L soft agar were used for phage assays.

Mating procedure. After growth overnight in Penassay Broth, 10-ml portions of donor and recipient

cells were washed with 0.85% saline and suspended in 1.0 ml of saline. At this time, 0.1 ml of the male cell suspension and 0.1 ml of the female cell suspension were plated separately (as controls) and together (for mating) on appropriate minimal selective agar. After incubation at 37 C for 2 days, recombinant colonies were picked and purified by streaking on the original selective medium.

Preparation of phage P1 lysates. P1 *vir* phage was grown on suitable *E. coli* K-12 and *S. flexneri* hosts by the soft agar layer method (1). Usually, 10 plates of L agar overlayer were prepared by adding enough P1 *vir* to host cells to yield almost confluent lysis. After overnight incubation at 37 C, 5.0 ml of L broth was added to each plate; the overlayers were scraped and macerated and then centrifuged to remove the agar and debris. The resulting supernatant fractions were pooled, sterilized by filtration (0.45 μm pore size; Millipore Corp., Bedford, Mass.), and preserved with chloroform (0.3 ml of $CHCl_3$ per 10 ml of lysate). The number of plaque-forming units per ml of lysate was determined by soft agar layer titrations.

Transduction procedure. Recipient strains were streaked on L agar and, after growth overnight, were harvested into 10 ml of L broth (about 10^{10} cells/ml). Phage P1 *vir* was added to a 5.0-ml portion to achieve the desired phage-bacterium input ratio (usually one phage per five cells). A control (5.0-ml sample) of cells was treated with L broth instead of with phage. After 20 min of incubation, the suspensions were treated with saline-citrate buffer, centrifuged, and resuspended in 0.5 ml of saline-citrate buffer. At this time, 0.1-ml portions were plated on appropriate selective medium.

Isolation of mutants. One-tenth milliliter of aqueous solution containing 400 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to 1 ml of a 10-fold concentrate of an overnight Penassay Broth culture of the parent strain. This was incubated for 25 min at 37 C, after which 4 ml of Penassay Broth was added. After 3 hr of additional incubation at 37 C, the culture was diluted, spread on nutrient agar plates, and incubated overnight. Mutants detected by replica plating to appropriate minimal media were repurified by streaking and were scored for their nutritional requirement.

Guinea pig keratoconjunctivitis test. Details of the guinea pig keratoconjunctivitis test have been described (13). Strains considered to be virulent enter the corneal epithelial cells and cause keratoconjunctivitis (10, 14). To insure against the possibility of false-negative reactions, broth suspensions of agar-grown bacteria containing 2×10^{10} cells per ml were employed. This is 10 to 100 times the number of bacteria usually required for a virulent strain to cause a positive reaction. A drop of this suspension was deposited into the conjunctival sac of a guinea pig, and the animal was observed daily for 72 hr. A reaction was considered to be positive if the cornea became opaque and conjunctivitis developed.

RESULTS

Intergeneric matings. For initial studies, we employed *S. flexneri* 2a strain 191b as a recipient and, as donors, several *E. coli* K-12 Hfr strains which differed in their point of origin and polarity of chromosome transfer (Fig. 1). The 191b recipient strain, virulent as judged by its ability to cause keratoconjunctivitis in guinea pigs, is a streptomycin-resistant, *gal*$^-$ mutant of *S. flexneri* strain M42-43 (Table 1). This recipient was mated with the various *E. coli* Hfr strains and selections were made for the following donor carbohydrate utilization markers: *lac*$^+$, *mal*$^+$, *fuc*$^+$, and *gal*$^+$. After 2 days of incubation, hybrid clones were purified by streaking on the original selective medium and were scored for the acquisition of nonselected markers and for their capacity to provoke keratoconjunctivitis. It soon became apparent (Table 2) that many hybrids which had inherited either the *lac* or *gal* chromosomal markers of *E. coli*, regardless of the Hfr used, also lost the capability to give a positive Sereney test. This is particularly evident with Hfr Hayes, Hfr KL96, and Hfr AB311, whose polarity of transfer result in strong linkage between the *lac* and *gal* markers (Fig. 1). With these donor strains, 100% of the hybrids in classes that inherited both the *lac* and *gal* loci failed to yield a positive keratoconjunctivitis test. In instances where the *S. flexneri* hybrids had inherited either the *gal* or *lac* locus, some lost and some retained their ability to cause keratoconjunctivitis. With W1895, which has a point of origin between the *lac* and *gal* loci (hence resulting in no detectable genetic

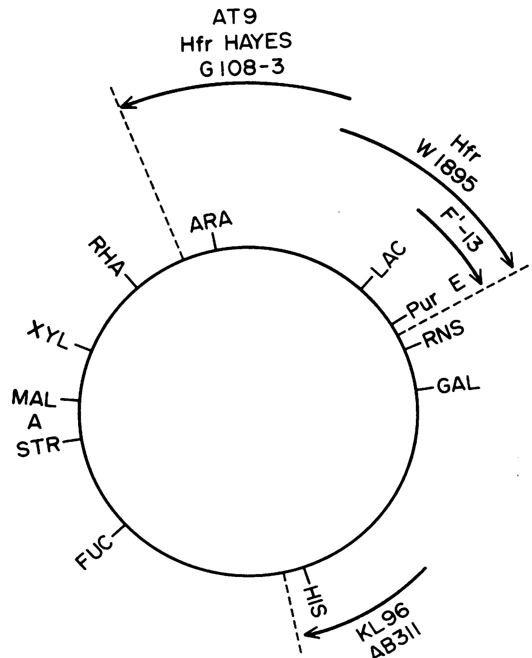

FIG. 1. *Escherichia coli K-12 chromosome.*

TABLE 1. *Bacterial strains*[a]

Organism	Mating polarity	Utilization of							Auxotrophic characters									
		lac	*ara*	*rha*	*xyl*	*mal*	*fuc*	*gal*	*purE*	*thi*	*met*	*nic*	*asp*	*his*	*thr*	*leu*	*rns*	*str*
Shigella flexneri 2a																		
M-42-43	F⁻	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+	+	S
S. flexneri 2a 191b	F⁻	−	−	−	−	−	−	−	+	+	+	−	−	+	+	+	+	R
S. flexneri 2a G256-2	F⁻	−	−	−	−	−	−	−	−	+	+	−	−	+	+	+	+	S
Escherichia coli K-12																		
W1895	Hfr	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	S
E. coli K-12 Hayes	Hfr	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	S
E. coli K-12 G108-3	Hfr	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	S
E. coli K-12 AT9	Hfr	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	S
E. coli K-12 AB311	Hfr	+	+	+	+	+	+	+	+	−	+	+	+	+	−	−	+	R
E. coli K-12 KL96	Hfr	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	S

[a] Abbreviations: *lac*, lactose; *ara*, arabinose; *rha*, rhamnose; *mal*, maltose; *fuc*, fucose; *gal*, galactose; *pur*, purine, *thi*, thiamine; *met*, methionine; *nic*, nicotinic acid; *asp*, aspartic acid; *his*, histidine; *thr*, threonine; *leu*, leucine, *rns*, ribonuclease; *str*, streptomycin; S, sensitive; R, resistant.

linkage between them), all of the hybrids tested remained unchanged in their capacity to provoke a positive Sereney test.

Serological tests indicated that all of the *lac*⁺-*gal*⁺ hybrids had lost the type-specific II antigen. Since it has been previously shown that gene(s) controlling the type-specific II antigen of *S. flexneri* are linked to the *lac* operon (7, 12), this change in serological properties after hybridization with the *E. coli lac* chromosomal region was anticipated. Retention of type II antigenic specificity by hybrids, however, was not necessary for the maintenance of virulence, because most of the *lac*⁺ *gal*⁻ hybrids which remained virulent had lost such specificity.

This preliminary screening of various *S. flexneri* hybrid classes clearly indicated that a locus responsible for virulence, as operationally defined by the Sereney test, is genetically linked to the *lac-gal* chromosomal segment.

To position further the *kcpA* locus, we sought to isolate by NTG mutagenesis a derivative of Hfr H which was mutated in the *purE* locus, this marker being located in the *lac-gal* chromosomal segment (17). One of the mutants, termed G108-3, behaved sexually as a typical Hfr H, and in addition to requiring adenine for growth was also *thi*⁻. We next established that the adenine requirement of G108-3 was due to a mutation in the *purE* locus. Hfr G108-3 was mated with *S. flexneri* 191b recipients, and selections were made for donor *lac*⁺ and *gal*⁺ markers on medium containing or lacking adenine. Hybrids which had or had not inherited the *ade*⁻ locus of the G108-3 donor could thus be studied (Table 3). In general, when selections were made for *lac*⁺ or *gal*⁺ hybrids in the presence or absence of adenine, the frequency of recombination was approximately 10^{-5}, expressed as the number of hybrids per donor cell. The high genetic linkage of the donor *ade*⁻ locus to *gal* and *lac* loci led us to the conclusion that G108-3 is mutated in its *purE* locus. This conclusion was supported by cross-over analysis which positioned this mutation between the *lac* and *gal* loci, a region previously assigned for *purE* (16). Furthermore, episomal transfer of the F′13 merogenote, which encompasses the *purE* locus to G108-3, resulted in adenine independence.

Hybrids of different genotypic classes derived from matings of *E. coli* Hfr G108-3 with *S. flexneri* 191b were next tested for their ability to cause keratoconjunctivitis. It is evident (Table 4) that, whenever the *purE*⁻ locus of *E. coli* is incorporated into the *S. flexneri* genome, the resultant hybrids are uniformly avirulent by the Sereney test (see hybrid classes B, C, and F). Moreover, hybrid classes that have retained their native *S. flexneri purE*⁺ allele (see classes A, D, and E) for the most part retain their ability to cause keratoconjunctivitis. On the basis of these findings, we conclude that the *kcpA* locus, concerned with epithelial cell penetration of *S. flexneri*, is closely linked to the *purE* allele.

It is well known that purine-requiring stains of *Salmonella typhosa* and other enteric bacteria are often avirulent (9, 10–13). Such avirulence results from an inability of infecting cells to grow in the peritoneal cavity of the mouse, which is lacking in available purines. If such strains are reverted to purine independence, there usually is a concomitant return to virulence. In light of these reports, it became essential to establish that the purine requirement was not in itself responsible for the loss of virulence of the *purE*⁻ hybrid

TABLE 2. *Ability of Shigella flexneri 191b hybrids derived from matings with various Escherichia coli K-12 strains to cause keratoconjunctivitis*

E. coli K-12 donor	Donor selected character[b]	Hybrid class[a]		No. analyzed	Keratoconjunctivitis	
		lac	*gal*		No. positive	No. negative
Hfr Hayes	*lac*	+	−	9	6	3
	lac	+	+	17	0	17
	mal	−	−	10	10	0
	fuc	−	−	10	9	1(±)
	fuc	+	+	2	0	2
	gal	−	+	7	2	5
	gal	+	+	15	0	15
AB311	*mal*	−	+	3	1	2
	mal	+	+	6	0	6
	fuc	−	−	9	9	0
	fuc	−	+	1	0	1
	gal	−	+	17	0	17
	gal	+	+	21	0	21
KL96	*lac*	+	−	19	18	1
	lac	+	+	5	0	5
	gal	−	+	14	7	7
	gal	+	+	17	0	17
W1895	*lac*	+	−	15	15	0
	mal	−	−	17	17	0
	xyl	−	−	9	9	0
	fuc	−	−	16	16	0
	fuc	+	−	2	2	0

[a] For simplicity and convenience of correlating the Sereney test results, hybrid classes have been differentiated on the basis of their incorporation of the unselected markers *lac* or *gal*, or both.

[b] Abbreviations: *lac*, lactose; *mal*, maltose; *fuc*, fucose; *gal*, galactose; *xyl*, xylose.

strains. For this reason, concentrated suspensions of three different *purE*$^-$ hybrids were plated on medium lacking purine, and a total of 12 purine-independent (*purE*$^+$) reverted clones were isolated. All 12 reverted strains, when tested by the Sereney test, remained avirulent. An alternative approach for separating genetically avirulence and purine dependence yielded a similar conclusion. In this instance, virulent *S. flexneri* strain M42-43 was treated with the mutagen NTG, and purine-requiring strains, mutated in the *purE* locus (strain G252-2) as well as in other *pur* loci, were recovered. Such hybrids retained their virulence despite their purine dependence for growth. Further support for the conclusion that purine dependence and loss of virulence are separable came from the detection of purines in the fluids of the conjunctival sac. Sterile paper discs were placed into the conjunctival sac of anesthetized guinea pigs. After saturation with fluids, they were transferred to minimal medium agar, lacking adenine, which had been previously inoculated with washed suspensions of *purE*$^-$ *S. flexneri* strains. After overnight incubation, distinct zones of growth by the purine-dependent strains were readily detected.

Transduction of the kcp virulence locus by phage P1 vir. Because conjugation experiments revealed high genetic linkage between the *purE* locus and the *kcpA* marker, we next performed transduction experiments with phage P1 *vir* to determine whether these two loci were cotransducible. Phage P1 *vir* was grown on wild-type *purE*$^+$ *kcpA*$^+$ virulent *S. flexneri* 2a strain M42-43 and on avirulent *purE*$^+$ *kcpA*$^-$ *E. coli* K-12. These lysates were employed in transductions to *S. flexneri* avirulent hybrid strain G108-1-13 (*purE*$^-$), selection being made for *purE*$^+$ transductants. The resulting transductants were then tested for their ability to evoke keratoconjunctivitis. Of 48 clones transduced to purine independence by phage grown on *S. flexneri* M42-43, 30 (62%) caused a positive Sereney test, indicating that the *kcpA* locus is cotransduced with the

TABLE 3. *Unselected marker analysis of hybrids derived from matings of Escherichia coli K-12 Hfr G108-3 and Shigella flexneri 191b*

Selection	Adenine supplement	No. tested	No. of hybrids with donor marker		
			lac$^+$	*gal*$^+$	*ade*$^-$
lac	−	63	63	46	0
lac	+	58	58	48	50
gal	−	41	2	41	0
gal	+	73	69	73	72

TABLE 4. *Ability of different Shigella flexneri 2a 191b hybrid classes to cause keratoconjunctivitis*

Hybrid class	Donor inherited markers			No. tested	Keratoconjunctivitis	
	lac$^+$	*pur E*$^-$	*gal*$^+$		No. positive	No. negative
A	+[a]	−[b]	−	19	16	3
B	+	+	−	3	0	3
C	+	+	+	89	0	89
D	+	−	+	24	20	4
E	−	−	+	32	27	5
F	−	+	+	7	0	7

[a] Inheritance of donor marker (+).

[b] Noninheritance of donor marker (−).

purE locus. Of 12 clones transduced by P1 grown on *E. coli* K-12, none caused keratoconjunctivitis.

Genetic analysis of a purE⁻ virulent S. flexneri. During the course of these studies, we isolated a virulent *purE⁻ S. flexneri* mutant (strain G252-2) for the purpose of separating genetically the virulence phenotype from the purine-dependent phenotype. This *S. flexneri* strain was now mated with Hfr strain AT9, (*met-*, ribonuclease⁻) selections being made for adenine independence (*purE⁺* donor alleles) and lactose utilization (*lac⁺* donor alleles). The results of Sereney tests performed with different hybrid classes are presented in Table 5. Hybrid classes which retained *purE⁻* chromosomal segment of the virulent recipient were found to remain virulent (class D). In contrast, hybrids which incorporated the *purE⁺* donor segment (classes A, B, C, E, and F) were, for the most part, avirulent (4 of 52 retained virulence).

We also carried out experiments which involved the transduction of mutant *S. flexneri* 2a strain 256-2 (*purE⁻*) to purine independence by using P-1 phage propagated on either *E. coli* K-12 or *S. flexneri* strain M42-43. Nineteen of 62 clones (34%) transduced to purine independence by P1 grown on *E. coli* were avirulent, whereas all of 10 clones similarly transduced by phage grown on virulent *S. flexneri* caused keratoconjunctivitis.

Incorporation of the S. flexneri 2a kcpA locus into an E. coli Hfr strain. The ability to cotransduce *kcpA* with the *purE* marker of *S. flexneri* suggested the possibility of constructing an *E. coli* Hfr strain which contains an *S. flexneri kcpA* locus. A lysate of phage P1 *vir*, prepared on virulent *purE⁺ kcpA⁺ S. flexneri* M42-43, was employed to transduce the *purE⁺* allele to *E. coli* Hfr Hayes *purE⁻*. Four *purE⁺* transductants of this Hfr were then tested to determine whether they had been cotransduced for the *S. flexneri kcpA⁺* locus. Since these transductants failed to provoke keratoconjunctivitis, genetic analyses were used to determine whether the *kcpA* locus was present. These derivatives were mated with virulent *S. flexneri* 191-B *gal⁻*, and five *gal⁺* hybrids from each cross, which also inherited the unselected *E. coli lac⁺* locus, were tested for their ability to provoke a positive Sereney test. Previous studies on this class of hybrid, prepared by mating *E. coli* Hfr H with virulent *S. flexneri* 191b, revealed this class to be uniformly avirulent (*see* Table 4). Of the four *purE⁺* transductants of Hfr Hayes tested by this mating procedure, three yielded *lac⁺ gal⁺* 191b hybrids which were uniformly avirulent (none of five hybrids from each mating gave a positive Sereney test) and thus appeared not to have the *S. flexneri kcpA⁺* locus after transduction of the *S. flexneri purE⁺* allele. In contrast, seventeen of seventeen *lac⁺ gal⁺* hybrids, derived from a mating with Hfr Hayes *purE⁺* transductant 4, retained their virulence, thus suggesting that this Hfr Hayes derivative had been cotransduced for the *kcpA⁺* locus of *S. flexneri.* Unequivocal evidence for the presence of this locus in *purE⁺* transductant 4 was established by mating this donor with an avirulent *S. flexneri* hybrid recipient (*purE⁻*, *kcpA⁻*). Hfr Hayes *purE⁺* transductant 4 and, as a control, Hfr Hayes AT9 were mated with the avirulent *S. flexneri* recipient, and selections were made for *purE⁺* hybrids. With the Hfr Hayes AT9 donor, none of 10 *purE⁺* hybrids provoked a positive keratoconjunctivitis, whereas, with the *purE⁺* transductant 4 derivatives of Hfr Hayes, five of seven *purE⁺* hybrids became virulent. We thus conclude, on the basis of such genetic transfer, that this derivative of Hfr Hayes has as part of its genotype the *kcpA⁺* locus of *S. flexneri* 2a.

TABLE 5. *Analysis of hybrids derived from mating Escherichia coli Hfr H AT-9 X Shigella flexneri G252-2 purE⁻ virulent*

Hybrid class	Donor marker inheritance			No. tested	Keratoconjunctivitis	
	lac⁺	RNAase[a]	*purE⁻*		No. positive	No. negative
A	+[b]	−[c]	+	27	1	26
B	+	+	+	6	0	6
C	−	+	+	11	1	10
D	+	−	−	16	16	0
E	+	−	+	21	2	19
F	−	+	+	1	0	1

[a] Ribonuclease⁻.
[b] Inheritance of donor marker (+).
[c] Noninheritance of donor marker (−).

DISCUSSION

The principal pathological feature of classical bacillary dysentery is the ulcerative lesion of the intestinal mucosa through which red blood cells and inflammatory cells pass into the feces. An initial step in the formation of the ulcer is the penetration of intestinal epithelial cells by the dysentery bacillus. Strains of dysentery bacilli which are unable to penetrate the epithelial barrier fail to cause disease (10; Schneider and Formal, Bacteriol. Proc., p. 66, 1963).

We previously employed intergeneric conjugation between *E. coli* K-12 Hfr donors and virulent *S. flexneri* recipients to prepare hybrids which are altered in their virulence (4). In such studies, vurulence was measured by the ability of hybrids to cause a fatal enteric infection in starved

or carbon tetrachloride-treated guinea pigs. After investigation of hybrid classes of *S. flexneri* containing segments of *E. coli* chromosome covering about 50% of the genome, only those which incorporated the *E. coli xyl-rha* chromosomal region were consistently altered to virulence, failing to cause death of infected animals. Subsequent studies showed that the loss of virulence was caused not by an inability of hybrid cells to penetrate the epithelial cells of the intestinal mucosa but, rather, by an inability of the bacterial cells to multiply sufficiently in epithelial layers after penetration (8). Thus, this *S. flexneri* hybrid still retained its property of penetrating mucosal cells, the primary step in the pathogenesis of bacillary dysentery. In the present communication, we have summarized experiments concerned with this essential property of virulent *S. flexneri* and have identified a genetic locus on the *S. flexneri* chromosome which controls epithelial cell penetration. Unlike our previous work, in which we employed the guinea pig model to assess virulence, the present study is based on the use of the Sereney test for keratoconjunctivitis as a model for detecting epithelial cell penetration. Our experience from previous work indicated that the guinea pig model is not suited for testing a wide variety of strains. We therefore chose the Sereney test because of its convenience in screening large numbers of strains and also because the results obtained from it correlate well with the ability of strains to penetrate the intestinal epithelium as revealed by fluorescent antibody microscopy.

The present investigation establishes a locus on the genome of *S. flexneri* which controls its ability to evoke a positive Sereney test. The locus, which we have termed *kcpA* in reference to its involvement in provoking keratoconjunctivitis, has been mapped between the *lac* and *gal* chromosomal markers in close proximity to the *purE* allele. This conclusion is based on both conjugational and transductional analyses. We observed that *S. flexneri* 2a hybrids which received the *lac-gal* chromosomal segment from various *E. coli* K-12 Hfr derivatives lost the ability to evoke a positive Sereney test. Further genetic analyses with P1 *vir* transduction experiments indicated that the *kcpA* locus was cotransducible with the *purE* allele. Thus it was possible to perform reciprocal transductions for the purpose of cotransducing, with the selected $purE^+$ marker, either the $kcpA^-$ allele of *E. coli* K-12 or the $kcpA^+$ allele of *S. flexneri* 2a. Transduction to *S. flexneri* $purE^-$ $kcpA^+$ with lysates of P1 *vir* grown on $purE^+$ $kcpA^-$ *E. coli* K-12 yielded a significant proportion (38%) of $purE^+$ transductants which also inherited the $kcpA^-$ allele, thus becoming unable to evoke keratoconjunctivitis. Likewise, in transductions to avirulent *S. flexneri* $purE^-$ $kcpA^-$ recipients with lysates propagated on virulent $purE^+$ $kcpA^+$ *S. flexneri* M42-43, a high proportion (68%) of the $purE^+$ transductants regained the ability to cause keratoconjunctivitis (i.e., became $kcpA^+$). Indeed, by such transduction procedures we have been able to construct an *E. coli* Hfr Hayes derivative which has as part of its genome the $kcpA^+$ locus of *S. flexneri*. This *E. coli* strain does not penetrate corneal epithelial cells, thus supporting our suspicion that other loci are involved in the control of this process. The presence of the $kcpA^+$ allele in this *E. coli* Hfr donor could, however, be readily demonstrated by genetic transfer to appropriate $kcpA^-$ recipients.

Since many of the hybrids which lost their ability to cause a positive Sereney test had also acquired a purine requirement for growth, we were concerned with the possibility that purine dependence could be related to the failure to penetrate cells. Several years ago we and others demonstrated that purine-requiring strains of a variety of bacterial species were avirulent for laboratory models if purine was absent at the site of inoculation, usually the peritoneal cavity (2, 3, 5, 6, 9). Reversion to purine independence restored the virulence of these organisms for these models. The route of infection obviously is important. For instance, purine-requiring strains of *Vibrio cholerae* are avirulent if injected intraperitoneally, because in this region purines are not present in sufficient amounts to support bacterial multiplication. However, when fed to experimental animals, these same strains may be just as pathogenic as purine-independent organisms, since purines are present in the intestines in sufficient amounts to support growth (6).

At this stage of the present study, the requirement for purine on the part of our hybrids did not appear to be responsible for the loss of virulence (i.e., cell penetration) for the following reasons. First, fluid from the conjunctival sac of guinea pigs adsorbed on filter paper discs stimulated the growth of purine-dependent organisms, thus indicating that purines are present in conjunctival fluids. Secondly, avirulent hybrids did not become virulent when they reverted to the purine-independent state. Likewise, a purine-requiring hybrid which was transduced to purine independence by P1 phage grown on *E. coli* remained avirulent, whereas the same hybrid regained its ability to cause keratoconjunctivitis if transduced to purine independence by P1 phage grown on virulent wild-type *S. flexneri* 2a.

This concern over separating the requirement for purine per se from virulence was obviated when

we isolated purine-requiring *S. flexneri* 2a mutants which maintained their potential to cause keratoconjunctivitis. One such strain was shown to be defective in the *purE* locus by linkage analysis following recombination and by use of the F′13 episome carried by an *E. coli* K-12 strain. Thus, the situation with *S. flexneri* avirulence was exactly opposite from the results cited above as far as relationship between virulence and the requirement for purine is concerned.

At the present time, the nature of the alteration to *S. flexneri* cells which results in a loss of penetrating ability and, hence, virulence remains obscure. Because conjunctival fluid contains lysozyme, we considered the possibility that *S. flexneri* $kcpA^-$ avirulent hybrids were more sensitive to lysozyme digestion than virulent *S. flexneri*. Recent experiments, however, tend to exclude this hypothesis for explaining the loss of virulence. Unlike *E. coli* K-12 which is sensitive to lysozyme (17), $kcpA^-$, nonpenetrating *S. flexneri* hybrids appear as resistant to lysozyme as their virulent *S. flexneri* parent.

From the present study employing the Sereney test, only a single locus controlling epithelial cell penetration has been discovered. The behavior of these hybrids in regard to epithelial cell penetration is currently being investigated in other model systems. In addition, since *E. coli* K-12 hybridized with the $kcpA^+$ allele of *S. flexneri* do not evoke keratoconjunctivitis, other genetic loci must be involved in this process. Attempts to establish identity of other loci are being made. The knowledge of such loci potentially controlling virulence will prove useful in constructing safe, living, attenuated shigella vaccines.

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LITERATURE CITED

1. Adams, H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Bacon, G. A., T. W. Burrows, and M. Yates. 1950. The effects of biochemical mutation on the virulence of *Bacterium typhosum*: the virulence of mutants. Brit. J. Exp. Pathol. 31:714–724.
3. Bacon, G. A., T. W. Burrows, and M. Yates. 1951. The effects of biochemical mutation on the virulence of *Bacterium typhosum*: the loss of virulence of certain mutants. Brit. J. Exp. Pathol. 32:85–96.
4. Falkow, S., H. Schneider, L. S. Baron, and S. B. Formal. 1963. Virulence of *Escherichia-Shigella* genetic hybrids for the guinea pig. J. Bacteriol. 86:1251–1258.
5. Formal, S. B., L. S. Baron, and W. Spilman. 1954. Studies on the virulence of a naturally occurring mutant of Salmonella typhosa. J. Bacteriol. 68:117–121.
6. Formal, S. B., H. Bohner, H. Schneider, and L. S. Baron. 1960. Virulence of a nutritional mutant of *Vibrio comma*. Proc. Soc. Exp. Biol. Med. 103:359–361.
7. Formal, S. B., P. Gemski, Jr., L. S. Baron, and E. H. LaBrec. 1970. Genetic transfer of *Shigella flexneri* antigens to *Escherichia coli* K-12. Infec. Immun. 1:279–287.
8. 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.
9. Garber, E. D., A. J. Hackett, and R. Franklin. 1952. The virulence of biochemical mutants of *Klebsiella pneumoniae*. Proc. Nat. Acad. Sci. U.S.A. 38:693–697.
10. 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.
11. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology 1:190–206.
12. Luria, S. E., and J. W. Burrous. 1957. Hybridization between *Escherichia coli* and *Shigella*. J. Bacteriol. 74:461–476.
13. Mackel, D. C., L. F. Langley, and L. A. Venice. 1961. The use of the guinea pig conjunctivae as an experimental model for the study of virulence of *Shigella* organisms. Amer. J. Hyg. 73:219–223.
14. Piéchaud, M., S. Szturm-Rubensten, and D. Piéchaud. 1958. Evolution histologique de la kérato-conjonctivite a bacillus dysentériques du cobaye. Ann. Inst. Pasteur 94:298–300.
15. Serény, B. 1957. Experimental keratoconjunctivitis *Shigellosa*. Acta Microbiol. Hung. 4:367–376.
16. Taylor, A. L., and C. D. Trotter. 1967. Revised linkage map of *Escherichia coli*. Bacteriol. Rev. 31:332–353.
17. Zinder, N. D., and W. F. Arndt. 1956. Production of protoplasts of *Escherichia coli* by lysozyme treatment. Proc. Nat. Acad. Sci. U.S.A. 42:586–590.