JOURNAL OF BACTERIOLOGY, Mar., 1965
Vol. 89, No. 3

Restoration of Virulence to a Strain of *Shigella flexneri* by Mating with *Escherichia coli*

SAMUEL B. FORMAL, E. H. LABREC, H. SCHNEIDER, AND STANLEY FALKOW

Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, D.C.

Received for publication 16 October 1964

ABSTRACT

FORMAL, SAMUEL B. (Walter Reed Army Institute of Research, Washington, D.C.), E. H. LABREC, H. SCHNEIDER, AND STANLEY FALKOW. Restoration of virulence to a strain of *Shigella flexneri* by mating with *Escherichia coli*. J. Bacteriol. **89**:835–838. 1965. —Three spontaneous avirulent variants of *Shigella flexneri* 5 were isolated and employed as genetic recipients in matings with *Escherichia coli* K-12. The various hybrid classes isolated from these matings were subsequently examined for their ability to induce keratoconjunctivitis and to kill pretreated guinea pigs. All hybrids derived from two of the variants remained avirulent. A majority of the *mal*$^+$ hybrids of the third avirulent variant were observed to be restored to complete virulence.

Thus far, all studies indicate that *Shigella flexneri* exhibits some genetic homology with *Escherichia coli* K-12 (Luria and Burrous, 1957; Falkow et al., 1963; Schneider and Falkow, 1964). Reports from this laboratory have previously demonstrated that the substitution of certain critical chromosomal regions of the *Shigella* genome with *Escherichia* genetic material results in the loss of *Shigella* virulence for the pretreated guinea pig (Falkow et al., 1963). Our screening procedures, however, were based on the premise that none of the *Escherichia* genes could contribute to the virulent phenotype. Thus, any *E. coli* gene which could contribute to the virulence would not have been detected. Because the fully virulent phenotype appears to be a polygenic phenomenon, it is not unreasonable to expect that occasionally two phenotypically avirulent organisms could give rise to a fully virulent hybrid by genetic exchange.

Virulent *Shigella* strains segregate colonial variants some of which are characterized by a change in virulence for several animal models. During our investigations of *Shigella* genetics, three separate, but similar, avirulent colonial variants of *S. flexneri* 5 were isolated and employed as genetic recipients with various donor strains of *E. coli* K-12. The various hybrid classes isolated from these matings were subsequently examined for virulence in several experimental models. All hybrids derived from two of the variants were avirulent. However, complete restoration of virulence was consistently observed to segregate among certain hybrid classes of the third variant. The purpose of this communication is to summarize these studies.

MATERIALS AND METHODS

Bacterial strains. S. flexneri 5 strain M90 TX is a colonial variant of strain M90 initially isolated in Mexico City in 1955 from a child with dysentery. The *E. coli* K-12 strain W1895 was described previously (Falkow et al., 1963). The pertinent genetic characteristics of these strains are shown in Table 1.

Media. Meat extract-agar and Penassay Broth (Difco) were used for the routine cultivation of organisms. Minimal agar employed for the selection and purification of genetic hybrids was prepared as described by Falkow, Rownd, and Baron (1962).

Mating procedures. Cells from overnight broth cultures were adjusted to a cell density of about 5×10^8 cells per milliliter, and 2 ml of the *E. coli* donor were diluted into 6 ml of the *S. flexneri* recipient in a 125-ml Erlenmeyer flask. After incubation for 2 hr at 37 C, 0.1 ml of an appropriate dilution of the mating mixture was plated on a medium selective for hybrids. The donor strain was contraselected by omitting methionine from the selective medium. Except in those cases where selection for prototrophy was desired, nicotinic acid (2 μg/ml) and aspartic acid (20 μg/ml) were added to the selective media to satisfy the growth requirements of the *Shigella* recipient.

Oral infection of guinea pigs. Guinea pigs of the Hartley or Walter Reed strain, weighing 300 to 400 g, were employed. They were deprived of food for 4 days and were then fed, by stomach tube, 5×10^7 to 1×10^8 challenge organisms suspended in 10 ml of Brain Heart Infusion broth. Immedi-

TABLE 1. *Genetic characteristics of bacterial strains**

Organism	Auxotrophic characters			Utilization of						Mating polarity
	*met**	*nic*	*asp*	*lac*	*ara*	*rha*	*xyl*	*mal*	*fuc*	
Shigella flexneri 5 M90 TXF	+	−	−	−	−	−	−	−	−	F^-
Escherichia coli K-12 W1895	−	+	+	+	+	+	+	+	+	Hfr

* Abbreviations: *met*, methionine; *nic*, nicotinic acid; *asp*, aspartate; *lac*, lactose; *ara*, arabinose; *rha*, rhamnose; *xyl*, xylose; *mal*, maltose; *fuc*, fucose; + = synthesis or utilization; − = not synthesized or utilized; F^-, recipient; Hfr, high frequency of recombination donor.

ately after challenge, 1 ml of tincture of opium was injected intraperitoneally. The animals were observed for 3 days after challenge. Virulent strains produced ulcerative lesions of the bowel and subsequent death in a large proportion of the animals. Animals fed avirulent cells did not exhibit any bowel pathology and these animals survived (Schneider and Formal, 1963; LaBrec et al., 1964).

Guinea pig keratoconjunctivitis test. Details of the guinea pig keratoconjunctivitis test were described by Mackel, Langley, and Venice, (1961). Virulent strains cause keratoconjunctivitis; avirulent strains do not.

RESULTS

S. flexneri 5 strain M90 TXF arose as a spontaneous flat, transparent colonial variant among the characteristic translucent colonies of the virulent strain M90 on streaked plates of meat extract-agar. At the same time, two other morphologically similar variants, M90 TXG and M90 TXH, were also selected from other plates. Spontaneous reversion of these colonial mutants to the parental form has not been observed. These three *S. flexneri* variants, like *E. coli* K-12, were classified as avirulent by their inability to induce keratoconjunctivitis, or to kill pretreated guinea pigs. Moreover, by use of the technique of LaBrec et al. (1964), we found that all the variants had lost their ability to penetrate epithelial cells in tissue culture. Aside from this loss of virulence and change in colonial morphology, no nutritional, serological, or physiological differences were discernible between the variants and the M90 T parental strain. The appearance of avirulent colonial variants in virulent *S. flexneri* populations is not an unusual occurrence, and has been reported in detail by others (Cooper, Keller, and Walters, 1957; Watkins, 1960; LaBrec et al., 1964).

The three colonial variants were employed as genetic recipients in matings with the *E. coli* K-12 donor strain W1895. The various hybrid classes were screened for virulence by the keratoconjunctivitis test. All recombinant classes derived from M90 TXG and M90 TXH were uniformly avirulent. However, several hybrid classes of these two variants exhibited a return to the characteristic colonial morphology of M90. On the other hand, most of the M90 TXF hybrids which had incorporated a genetic segment carrying the *E. coli* maltose (*mal*$^+$) gene had regained the ability to cause keratoconjunctivitis in guinea pigs (Table 2). An occasional hybrid which had retained the *mal*$^-$ locus of *Shigella* but had incorporated the genes governing the fermentation of fucose (*fuc*$^+$) (*see* Falkow et al., 1963) were also positive. Most, but not all, of the hybrids which returned to virulence regained the colonial appearance of the parent strain, M90.

The specificity of *Escherichia* genetic material in affecting virulence repair was indicated by studies of hybrids which carried a full complement of *Shigella* genes and the critical *E. coli mal*$^+$ region as a persistent exogenote (partial diploids). Such hybrids continually segregate clones which have lost the *E. coli* genetic material and have returned to the haploid state. When tested for ability to produce keratoconjunctivitis, the partial diploids were found to possess this capacity, whereas the haploid segregants did not (Table 3).

A selected hybrid clone, FWM1, which had

TABLE 2. *Restoration of the ability to cause keratoconjunctivitis in a strain of Shigella flexneri 5 by genetic recombination with Escherichia coli K-12**

Hybrid classes derived from cross *E. coli* K-12 × *S. flexneri* 5 M90 TXF	No. of virulent clones/ no. of clones tested
lac$^+$ *ara*$^+$	0/12
ara$^+$	0/5
xyl$^+$	0/5
mal$^+$	5/6
fuc$^+$	1/8
nic$^+$	0/3
mal$^+$ *xyl*$^+$	3/3
lac$^+$ *ara*$^+$ *mal*$^+$	3/3
lac$^+$ *nic*$^+$	0/2
lac$^+$ *nic*$^+$ *mal*$^+$	1/1
fuc$^+$ *nic*$^+$	2/4
ara$^+$ *mal*$^+$	6/7

* Parents: *E. coli* K-12 variant W1895 (avirulent) and *S. flexneri* 5 variant M90 TXF (avirulent).

TABLE 3. *Ability of partial diploid hybrids of Shigella flexneri 5 and their haploid segregants to produce keratoconjunctivitis in the guinea pig*

Strain no.	Genotype of diploid	Genotype of haploid segregant	No. of animals exhibiting keratoconjunctivitis/no. of animals examined	
			Diploid	Haploid segregant
FWX19	$\frac{ara^+\ mal^+\ xyl^+}{ara^-\ mal^-\ xyl^-}$	$ara^-\ mal^-\ xyl^-$	4/4	0/4
FWX23	$\frac{ara^+\ mal^+\ xyl^+\ lac^+}{ara^-\ mal^-\ xyl^-\ lac^-}$	$ara^-\ mal^-\ xyl^-\ lac^-$	4/4	0/4

TABLE 4. *Ability of parent and hybrid strains to produce a fatal enteric infection and intestinal pathology in starved guinea pigs*

Challenge strain	Genotype	Deaths/total	Intestinal pathology/no. of animals examined
Shigella flexneri 5 M90 TXF	$lac^-\ ara^-\ mal^-\ rha^-\ xyl^-\ fuc^-\ nic^-$	0/6	0/8
Escherichia coli K-12 W1895	$lac^+\ ara^+\ mal^+\ rha^+\ xyl^+\ fuc^+\ nic^+$	0/6	0/4
Hybrid FWM1	$lac^-\ ara^-\ mal^+\ rha^-\ xyl^-\ fuc^-\ nic^-$	7/11	7/8

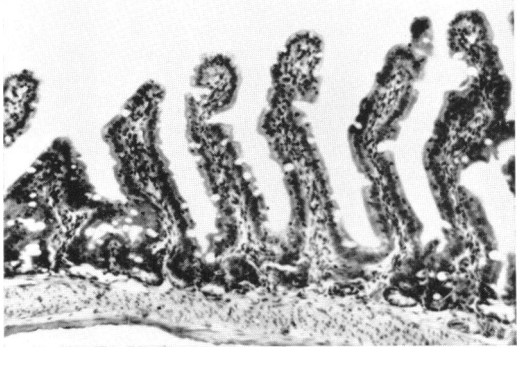

FIG. 1. *Terminal ileum of starved guinea pig 24 hr after the oral administration of Hfr Escherichia coli strain W1895. The epithelium, lamina propria, and villus architecture are normal. Hematoxylin and Eosin (H and E). ×136.*

regained the capacity to produce keratoconjunctivitis was also tested for its ability to cause intestinal pathology and a subsequent fatal infection in starved guinea pigs. Animals fed either the parental strains or the hybrid strain were killed 24 hr after infection, and specimens of intestinal tract were removed for histological examination. Other groups of animals similarly challenged were observed, to determine the incidence of mortality. These results are summarized in Table 4 and Fig. 1, 2, and 3. Clearly, the hybrid strain can induce intestinal pathology and cause the death of guinea pigs, whereas the parental strains can not.

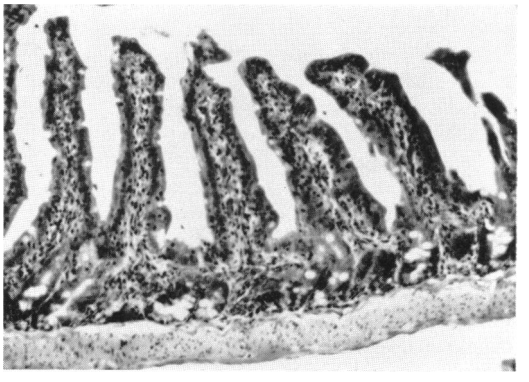

FIG. 2. *Terminal ileum of starved guinea pig 24 hr after the oral administration of recipient Shigella flexneri strain M90 TXF. The epithelium, lamina propria, and villus architecture are normal. H and E. ×132.*

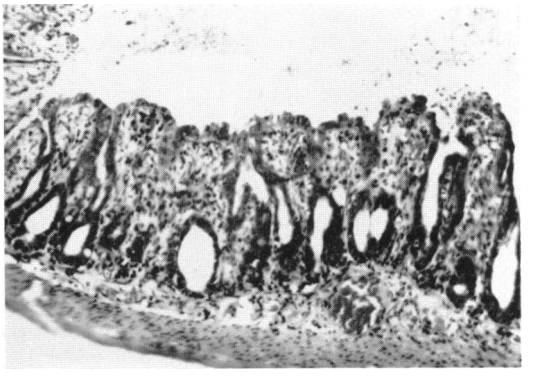

FIG. 3. *Terminal ileum of starved guinea pig 24 hr after the oral administration of Escherichia coli–Shigella flexneri hybrid strain FWM1. The epithelium is damaged, there is an acute inflammatory reaction in the lamina propria, and the villi are blunted, shortened, and fused. H and E. ×136.*

DISCUSSION

Other investigators have described colonial variants of *Shigella* strains which are associated with a loss of virulence for various animal hosts (Cooper et al., 1957; Kerekes, 1962). Our results indicate that the genetic basis for such a disappearance of virulence may differ from variant to variant. Thus, of three spontaneous avirulent mutants of strain M90 exhibiting the same discernible change in colonial morphology, only one, M90 TXF, could be restored to virulence by mating with *E. coli*. In a number of cases, we observed hybrids with the colonial morphology typical of the virulent M90 strain but without a concomitant return of virulence. Apparently, the determinant for colonial morphology is distinct from the one determining avirulence. It is clear that it is difficult to consider any single gene or function as being the sole determinant associated with the avirulent (or virulent) phenotype.

LaBrec et al. (1964) proposed that penetration of the intestinal epithelial cell, entrance to the lamina propria, and multiplication in this region are the sequence of events which occur when dysentery bacilli form ulcerative lesions of the intestinal mucosa. Cell penetration and at least limited survival in the lamina propria may be the minimal necessary attributes of shigellae which set them apart from nonpathogenic *E. coli* strains and avirulent shigellae. In the present study, the avirulent variants of *S. flexneri* 5 strain M90 all appeared to harbor a genetic defect which prevented them from penetrating the bowel epithelium. In the variant M90 TXF, this defect is repairable and permits the entire sequence to go to completion. That the genetic (and functional) sequence leading to penetration alone is complex is indicated by the observation that only one of three variants lacking penetration ability can be restored by mating.

The major disadvantage in employing *E. coli* for this study lies in the fact the *Escherichia* genes can cause virulence defects, as well as repair, in *S. flexneri* (Falkow et al., 1963). The recent isolation of *Shigella* genetic donors (Schneider and Falkow, 1964) should permit more definitive studies along these lines. Recently, we have also discovered avirulent derivatives of shigellae which retain the ability to penetrate the lamina propria, but which fail to survive for any significant period after penetration. We can reasonably hope that it will be possible to isolate a series of avirulent shigellae (either spontaneously or by mating) which are "blocked" at different steps in the infection process.

Most of our definitive knowledge of virulence is derived from the study of variants such as we have employed in this study. In the present case, the observed variation, which may only involve a single mutational event, is associated with ability to penetrate cells; however, mere association is not a demonstration of primary significance, especially in regard to virulence (Miles, 1955). Obviously, if the gene(s) involved is primarily concerned with cell penetration, it is quite significant, from the standpoint of the evolution of pathogenesis, that *E. coli* K-12 possesses at least one genetic determinant which can contribute to the expression of virulence.

LITERATURE CITED

COOPER, M. L., H. M. KELLER, AND E. W. WALTERS. 1957. Microscopic characteristics of colonies of Shigella flexneri 2a and 2b and their relation to antigenic composition, mouse virulence and immunogenicity. J. Immunol. **78**:160–171.

FALKOW, S., R. ROWND, AND L. S. BARON. 1962. Genetic homology between *Escherichia coli* K-12 and *Salmonella*. J. Bacteriol. **84**:1303–1312.

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.

KEREKES, L. 1962. Colonial variants of *Shigella flexneri*. Acta Microbiol. Acad. Sci. Hung. **9**:123–132.

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.

LURIA, S. E., AND J. W. BURROUS. 1957. Hybridization between *Escherichia coli* and Shigella. J. Bacteriol. **74**:461–476.

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. Am. J. Hyg. **73**:219–223.

MILES, A. A. 1955. The meaning of pathogenicity. Symp. Soc. Gen. Microbiol. **5**:1–16.

SCHNEIDER, H., AND S. FALKOW. 1964. Characterization of an Hfr strain of *Shigella flexneri*. J. Bacteriol. **88**:682–689.

SCHNEIDER, H., AND S. B. FORMAL. 1963. Spontaneous loss of guinea pig virulence in a strain of *Shigella flexneri* 2a. Bacteriol. Proc., p. 66.

WATKINS, H. M. S. 1960. Some attributes of virulence in *Shigella*. Ann. N.Y. Acad. Sci. **88**:1167–1186.