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ALTERATIONS IN THE MOUSE VIRULENCE OF *SALMONELLA TYPHIMURIUM* BY GENETIC RECOMBINATION

V. KRISHNAPILLAI[1] AND L. S. BARON

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

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ABSTRACT

KRISHNAPILLAI, V. (Walter Reed Army Institute of Research, Washington, D.C.), AND L. S. BARON. Alterations in the mouse virulence of *Salmonella typhimurium* by genetic recombination. J. Bacteriol. 87:598-605. 1964.—The genetic basis of mouse virulence was investigated with an avirulent strain of *Salmonella abony* as chromosomal donor and a virulent strain of *S. typhimurium* as recipient in recombination experiments. In these genetic crosses, the transfer of partial avirulence was found to segregate among the hybrids that were examined. At least two determinants controlling avirulence were depicted to account for the partial avirulence of the hybrids. One of these determinants is indicated as being in the region of the locus for streptomycin sensitivity or resistance, and the other was adjacent to the locus for inositol utilization. Moreover, both determinants were essential for the phenotypic expression of complete avirulence in a hybrid. This was established by the results of experiments in which an initial, partially avirulent hybrid was backcrossed with the *S. abony* donor so that it further received the additional determinant.

In recent years, a number of investigators have developed genetic systems in *Salmonella typhimurium* which make it possible to study extensive regions of the bacterial chromosome by recombination. The mechanics of the process appear very similar to the now classical sexual conjugation system of *Escherichia coli* K-12 (Jacob and Wollman, 1961). Initially, *S. typhimurium* is sterile either with *E. coli* K-12 donors (Baron, Carey, and Spilman, 1959; Miyake and Demerec, 1959) or within itself (Zinder and Lederberg, 1952). However, strains capable of acting as recipients have been isolated either fortuitously (Baron et al., 1959) or from *Salmonella* strains possessing the mutator gene (Miyake, 1962). In addition, Zinder (1960) was able to transfer the *E. coli* sex factor, F, into *S. typhimurium* by the simple procedure of cultivation of the bacteria together. From such F-infected cultures he was able to isolate Hfr mutants. More recently, Mäkelä (1963) analyzed a similar situation in *S. abony*. A novel system in *S. typhimurium*, where recombination is mediated by colicinogenic factors, was also documented by Smith and Stocker (1962).

The laboratory mouse is very susceptible to infection with *S. typhimurium* when the bacteria are introduced into the peritoneum. The infective process simulates the clinical manifestations of typhoid disease in man, generally characterized by diarrhea, and culminates in the death of the animal. Such an end point is used as a relative measure of the lethality of the organisms. Many strains of the same species or related *Salmonella* are completely avirulent or possess intermediate levels of virulence. Since this characteristic is usually stable for any particular species of *Salmonella*, it was expected that a genetic basis for mouse virulence might be found.

In the present paper, we describe experiments performed with *S. typhimurium* which pertain to the genetic basis of mouse virulence.

MATERIALS AND METHODS

Bacterial strains. The bacterial stocks used in this investigation and their characteristics are shown in Table 1. The Hfr strain SW1444 is identical to SW1391 (Mäkelä, 1963, *personal communication*), and is the most stable of the donor strains of *S. abony* isolated by Mäkelä. The C5 strain of *S. typhimurium* is a wild-type, prototrophic strain which is highly virulent for the mouse (Krishnapillai, Reeves, and Rowley, 1963). Selection of mutants in the C5 strain was accomplished as follows. The histidine-requiring mutant was obtained by ultraviolet irradiation and subsequent penicillin selection by use of a technique similar to that of Adelberg and Myers

[1] Present address: Department of Microbiology, University of Adelaide, Adelaide, South Australia.

TABLE 1. *Bacterial strains used in the study of virulence**

Strain	Source	Auxotrophic markers				Energy source utilization			Response to streptomycin	Flagellar H antigens		Mating polarity
		his	*met*	*arom*	*ile*	*inos*	*rha*	*ara*	(*str*)	H_1	H_2	
S. abony SW1444	P. H. Mäkelä	+	−	−	+	+	+	+	r	b	e,n,x	Hfr
S. typhimurium												
C5 His^-	C5	−	+	+	+	−	−	+	s	i	1,2	F^-
C5 His^-Ara^-	C5 His^-	−	+	+	+	−	−	−	s	i	1,2	F^-
C5 Ile^-	C5	+	+	+	−	−	−	+	s	n.u.	n.u.	F^-

* Abbreviations: *his* = histidine; *met* = methionine; *arom* = phenylalanine + tyrosine; *ile* = isoleucine; *inos* = inositol; *rha* = rhamnose; *ara* = arabinose; n.u. = not used during this investigation; Hfr = high frequency of recombination donor; F^- = recipient; + = synthesized or utilized; − = not synthesized or utilized; r = resistant; s = sensitive.

(1953); the isoleucine-requiring mutant was obtained by the procedure reported by Loveless and Howarth (1959), with ethyl methane sulfonate (EMS) as the mutagenic agent; the arabinose-negative mutant of C5 His^- was also isolated by treatment with EMS, except that the mutants were concentrated by exposure to penicillin (Lederberg, 1950) in the presence of histidine, with arabinose as the sole carbon source.

Media. Penassay Broth and meat-extract agar (both from Difco) were employed for the routine cultivation of the bacteria. The minimal medium and Eosin Methylene Blue (EMB) medium, along with the concentrations of amino acid, carbohydrate, or streptomycin sulfate supplements, were previously described by Falkow, Rownd, and Baron (1962).

Endo medium (Difco) was supplemented with the required carbohydrate at a concentration of 1%. This preparation contains lactose, but, since all the strains in this study are lactose nonfermenters, the use of this medium for detection of the utilization of other added sugars was not impaired.

Semisolid agar medium for the enhancement of motility was of the following composition per liter of distilled water: gelatin (BBL), 80 g; peptone (Difco), 10 g; beef extract (Difco), 3 g; and Noble agar base (Difco), 4 g.

Replica plating. The method described by Lederberg and Lederberg (1952) was used.

Determination of H antigens. Bacterial cultures to be analyzed for their flagellar antigens were made highly motile by two consecutive passages through semisolid agar medium. The diagnostic procedure was identical to that of Edwards and Ewing (1961), except for the use of broth cultures that were not treated with formalin.

Mating procedure. Cultures (18-hr) of the bacteria to be mated were separately washed and adjusted to give an approximate donor to recipient ratio of 1:5. Samples of the donor and recipient were plated together on plates of selective media; as controls, they were singly plated on the same medium. Purification of recombinants was accomplished on the same medium used in initial selection.

Characterization of recombinants. Replica plating and inoculation into differential media were employed to determine the possible segregation of unselected markers. To test stability of the purified recombinants, single colonies were streaked on differential and selective media. This procedure was repeated two to three times by restreaking to fresh media. In addition, single clones from nonselective media also were replicated to differential media to detect the possible segregation of alleles from the donor or the recipient, or both.

Test animals. Male mice of the Bagg strain, weighing 16 to 22 g, were used throughout this study.

Virulence tests. Groups of ten mice were challenged intraperitoneally with 0.5-ml amounts of different dose levels of bacteria. At challenge, viable counts were made by spreading samples of suitable dilutions on meat-extract agar plates. Cumulative deaths were recorded daily for 28 days. The 50% lethal dose (LD_{50}) was estimated by the method of Reed and Muench (1938). A screening procedure for detecting loss of virulence

TABLE 2. *Gradient of transfer of markers from Salmonella abony Hfr SW1444 to S. typhimurium C5 F⁻ recipients**

Recombinants selected	Frequency per donor bacterium
ara⁺	1×10^{-5}
leu⁺	1×10^{-5}
his⁺	2×10^{-6}
ile⁺	4×10^{-7}
val⁺	2×10^{-7}
rha⁺	2×10^{-8}
inos⁺	2×10^{-9}
cys⁺	$<2 \times 10^{-9}$
arg⁺	$<2 \times 10^{-9}$

* Recombinants were selected by the technique described in Materials and Methods. *Met* and *arom* were used as contraselections against the donor. The frequencies refer to the approximate number of recombinants obtained under the conditions of mating. The abbreviations *leu*⁺, *val*⁺, *cys*⁺, and *arg*⁺ refer to leucine, valine, cystine, and arginine, respectively. These four single nutritional mutants of *S. typhimurium* C5 were only used in this experiment.

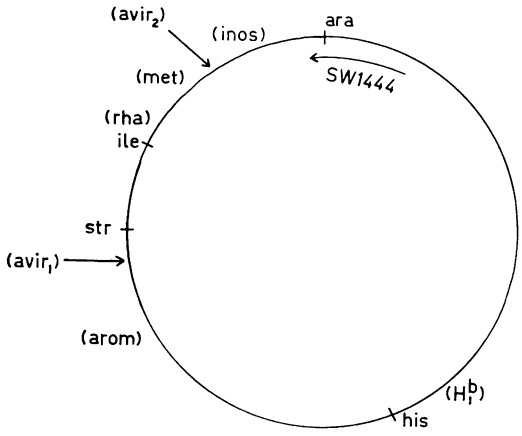

FIG. 1. *Chromosome map of Salmonella. The relative positions of the various loci are based on the Escherichia coli K-12 map (Jacob and Wollman, 1961) and also on the findings of Mäkelä (1963), Falkow et al. (1962), and Smith and Stocker (1962). The loci in parentheses indicate that the location of the marker in relation to the neighboring markers has not been determined precisely. The inner line refers to the order of transfer of markers by the Hfr donor; avir = avirulence determinants (see Discussion).*

with a single dose level of about 5×10^3 bacteria per mouse as the challenge level in groups of ten mice was employed also.

RESULTS

The data presented in Table 2 give an indication of the gradient of transfer of markers from *S. abony* into suitably marked recipients of *S. typhimurium* C5, and are compatible with the findings of Mäkelä (1963). In general, the genetic results obtained throughout this investigation are in essential agreement as to marker location and linkage pattern with those reported by Mäkelä (1963) and Falkow et al. (1962) in *S. typhosa* and *S. typhimurium*. The location of the markers used in this work is shown in Fig. 1.

Observations on the determination of virulence. The primary aim of this study was to determine whether the mouse virulence of the C5 strain of *S. typhimurium* could be modified by recombination with an avirulent *S. abony* strain. *S. abony* has the antigenic formula 4,5,12:b-e,n,x and *S. typhimurium* has the formula 1,4,5,12:i-1,2; thus, they are sufficiently alike antigenically to make meaningful comparisons of the change in virulence of the hybrids. The donor strain of *S. abony* is completely avirulent for the mouse (Table 3), being unable to multiply or produce infections in this experimental animal. It is lethal for mice only when a sufficiently high dose (about 10^8 bacteria) is injected to produce toxicity. Thus, when such a high dose level is used, a large proportion of mice succumb to the endotoxin effects within 2 to 3 days (endotoxic death), no further deaths occurring after this time period. As little as a fivefold reduction in the dose, however, produces very few, if any, deaths in the mice. The C5 strains of *S. typhimurium*, on the other hand, are highly virulent (Tables 3, 4, and 5). The mortality in groups of mice injected with even small doses of bacteria of the C5 strains is progressive with time, commencing 4 to 5 days after challenge and continuing until the termination of the experiment, usually 28 days. A characteristic difficulty in LD_{50} determinations with the C5 strains is that of obtaining satisfactory 50% end points. In the case of *S. typhimurium* C5 His⁻ Ara⁻ strain, for example, a challenge dose of 60 organisms produced death of seven of ten animals; 600 organisms resulted in death of six of ten animals; and

TABLE 3. *Virulence of hybrids obtained by recombination of Salmonella abony Hfr SW1444 and S. typhimurium C5 His⁻*

Bacterial strain	Selected marker of donor	Unselected marker(s) detected in hybrid	No. of hybrids tested	Virulent bacteria		Avirulent bacteria		Decrease in virulence*
				No.	LD_{50}	No.	LD_{50}	
S. abony Hfr SW1444 (donor)	—	—	—	—	—	—	1×10^8	50,000-fold
S. typhimurium C5 His⁻ (recipient)	—	—	—	—	2×10^3	—	—	—
Hybrid SW1444×C5 His⁻	*his*⁺	—	6	6	$\sim 5 \times 10^3$†	0	—	Unchanged
	his⁺	$H_1{}^b$	2	2	$\sim 5 \times 10^3$†	0	—	Unchanged
	his⁺	*str-r rha*⁺	2	0	—	2	2×10^5 2×10^5	100-fold 100-fold
	his⁺	*rha*⁺	3	3	$\sim 5 \times 10^3$†	0	—	Unchanged
	inos⁺	—	3	1	$\sim 5 \times 10^3$†	—	—	Unchanged
						2	2×10^5 2×10^5	100-fold 100-fold

* Expressed as the ratio of the LD_{50} of $\frac{\textit{S. abony}\text{ Hfr donor or hybrid}}{\textit{S. typhimurium}\text{ recipient}}$.

† The virulence status of these hybrids was determined by the virulence-screen test.

TABLE 4. *Virulence of hybrids obtained by recombination of Salmonella abony Hfr SW1444 and S. typhimurium C5 His⁻Ara⁻*

Bacterial strain	Selected marker of donor	Unselected marker detected in hybrid	No. of hybrids tested	Virulent bacteria		Avirulent bacteria		Decrease in virulence*
				No.	LD_{50}	No.	LD_{50}	
S. abony Hfr SW1444 (donor)	—	—	—	—	—	—	10^8	2,000,000-fold
S. typhimurium C5 His⁻Ara⁻(recipient)	—	—	—	—	$<5 \times 10^1$	—	—	—
Hybrid SW1444 × C5 His⁻Ara⁻	*his*⁺	*ara*⁺	3	3	$\sim 5 \times 10^3$†	0	—	Unchanged
	his⁺	*str-r*	1	0	—	1	10^5	2,000-fold

* Expressed as the ratio of the LD_{50} of $\frac{\textit{S. abony}\text{ Hfr donor or hybrid}}{\textit{S. typhimurium}\text{ recipient}}$.

† The virulent status of these hybrids was determined by the virulence-screen test.

6,000 organisms also yielded death of six of ten. While these results can be taken to indicate that the LD_{50} of the C5 strains is less than 50 organisms, occasionally a dose-response mortality curve is obtained from which the LD_{50} can be calculated, as in the case of the C5 His⁻ strain listed in Table 3. All the hybrids of partial avirulence which were studied, however, display a mortality curve that is dose-dependent.

Virulence screening. Instead of determining the LD_{50} of each of the many hybrids, a screening procedure for the initial detection of loss of virulence was used (see Materials and Methods). With this method, all hybrids still retaining virulence behaved in a similar manner to the original recipient strain of *S. typhimurium*. That is, with a challenge dose of approximately 5×10^3 bacteria, 50 to 100% mortality occurred in

TABLE 5. *Virulence of hybrids obtained by recombination of Salmonella abony Hfr SW 1444 and S. typhimurium C5 Ile⁻*

Bacterial strain	Selected marker of donor	Unselected marker(s) detected in hybrid	No. of hybrids tested	Virulent bacteria		Avirulent bacteria		Decrease in virulence*
				No.	LD_{50}	No.	LD_{50}	
S. abony Hfr SW1444 (donor)	—	—	—	—	—	—	1×10^8	2,000,000-fold
S. typhimurium C5 Ile⁻ (recipient)	—	—	—	—	$<5 \times 10^1$	—	—	—
Hybrid SW1444×C5 Ile⁻	*ile*⁺	—	20	19	$\sim 5 \times 10^3$†	—	—	Unchanged
						1	1×10^5	2,000-fold
	ile⁺	*rha*⁺	16	16	$\sim 5 \times 10^3$†	—	—	Unchanged
	ile⁺	*str-r*	6	2	$\sim 5 \times 10^3$†	—	—	Unchanged
							2×10^7	400,000-fold
						4	1×10^6	20,000-fold
							3×10^5	6,000-fold
							7×10^5	14,000-fold
	ile⁺	*str-r rha*⁺	1	0	—	1	6×10^5	12,000-fold
	ile⁺	*inos*⁺	1	1	$\sim 5 \times 10^3$†	0	—	Unchanged
	ile⁺	*rha*⁺ *inos*⁺	1	0	—	1	1×10^5	2,000-fold

* Expressed as the ratio of the LD_{50} of $\frac{\textit{S. abony}\text{ Hfr donor or hybrid}}{\textit{S. typhimurium}\text{ recipient}}$.

† The virulence status of these hybrids was determined by the virulence-screen test.

TABLE 6. *Virulence of hybrids obtained by backcrossing an initial hybrid Salmonella typhimurium C5 His⁺str-r with S. abony Hfr SW1444*

Bacterial strain	Selected marker of donor	Unselected marker(s) detected in hybrid	LD_{50}	Decrease in virulence*
S. abony Hfr SW1444 (donor)	—	—	1×10^8	2,000,000-fold
S. typhimurium C5 His⁺str-r (initial hybrid) as recipient	—	—	1×10^5	—
Backcross hybrid 1	*rha*⁺	—	3×10^6	60,000-fold
Backcross hybrid 2	*rha*⁺	H_1^b, *inos*⁺	1×10^8	2,000,000-fold
Backcross hybrid 3	*rha*⁺	*ara*⁺	8×10^5	16,000-fold

* Expressed as the ratio of the LD_{50} of $\frac{\textit{S. abony}\text{ Hfr donor or hybrid}}{\textit{S. typhimurium}\text{ C5 His}^-\text{Ara}^-\text{ recipient}}$.

these mice; hence, these hybrids were considered to be unchanged in their virulence characteristics. On the other hand, the *S. abony* donor, or any partially avirulent hybrid, produced 0 to 10% mortality with the same dose level of challenge organisms. Only those hybrids exhibiting a partial avirulence were retested to determine their LD_{50} values (Tables 3, 4, 5, and 6). The last column in Tables 3 to 6 expresses the decrease in virulence of the hybrids in relation to the virulent recipient strain of *S. typhimurium*, with an arbitrary measure of the decrease in virulence. In addition, the LD_{50} value of the *S. abony* Hfr strain is repeated for comparative purposes. All recombinants which showed a decrease in virulence by this screen test were examined further for the stability of the transferred genes, to ascertain whether they were unstable or had any of the attributes of segregating diploids observed in other crosses (Baron, Spilman, and Carey, 1960). The hybrids were all found to be stable, and segregation of any of the alleles was not observed.

Modification of virulence of S. abony-S. typhi-

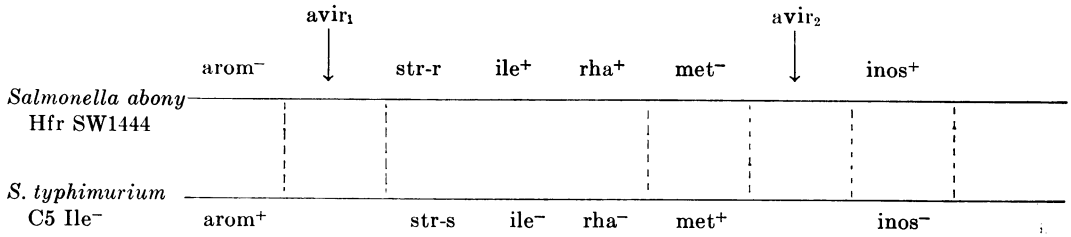

FIG. 2. *Presentation of the crossovers required for the formation of prototrophic or inos⁺ recombinants by genetic recombination, which could lead to the segregation of the avirulence determinants. Dashed lines indicate crossovers.*

murium hybrids. Table 3 shows that hybrids receiving the *his*⁺, *his*⁺$H_1{}^b$, or *his*⁺*rha*⁺ markers from *S. abony* are unchanged in their virulence. The transfer of either the *his*⁺*str*-r *rha*⁺ region or the *inos*⁺ marker by itself, however, produces partially avirulent hybrids. In Table 4 the transfer of the *his*⁺*ara*⁺ segment can be seen to have no effect on virulence, whereas the *his*⁺*str*-r region does affect virulence. These results suggest that the region involved in the determination of this difference in virulence lies within the chromosomal segment *his—str—inos* (see Fig. 1). The C5 Ile⁻ strain of *S. typhimurium*, therefore, was used as recipient in matings with the *S. abony* donor, to examine this region more closely. Various classes of hybrids isolated from this genetic cross were tested for their virulence pattern. The transfer of *ile*⁺ alone or *ile*⁺*rha*⁺ or *ile*⁺*inos*⁺ fail to produce any change in virulence (Table 5), therefore eliminating the individual segments from being associated with the observed virulence alteration. Those hybrids which possess the region *ile*⁺*str*-r, *ile*⁺*str*-r *rha*⁺, or *ile*⁺*rha*⁺*inos*⁺, however, exhibit a decrease in their virulence. Hence, an association of the loci for *str* and *inos* with the replacement of partial avirulence for full virulence by the C5 hybrids can be assumed. These loci are unlinked (Fig. 1 and 2).

It is obvious from an examination of Tables 3, 4, and 5 that the hybrids which display a loss in virulence do not possess the completely avirulent character of the *S. abony* donor. Therefore, additional crosses were performed to determine whether an initial, partially avirulent hybrid would become completely avirulent were it to further receive the other region involved in the transition of virulence to partial avirulence. For these studies, the hybrid C5 *his*⁺*str*-r (Table 4) possessing an LD_{50} of 10^5 bacteria was backcrossed with the *S. abony* Hfr, with selection being made for the *rha*⁺ marker of the donor. Table 6 illustrates the three genetic classes of backcross hybrids and the LD_{50} values obtained with these hybrids. The decrease in virulence expressed in this table is in terms of the original unmated C5 His⁻Ara⁻ strain of *S. typhimurium* and not by comparison with the initial partially avirulent recombinant. It is apparent that only the backcrossed hybrid which received the *inos* region, in addition to the previously transferred *str* region, becomes completely avirulent.

DISCUSSION

The results reported here demonstrate that the acquisition of partial avirulence is always associated with either the *str* or the *inos* marker, although it is clear that the reverse circumstance does not hold true. Evidently, a segregation of the partial avirulence characteristic occurs among hybrids possessing the *str* or *inos* marker. It becomes necessary, therefore, to look at the process of selection of these prototrophic recombinants to explain this segregation. The diagram in Fig. 2 illustrates the genetic aspects involved in this situation. The *S. abony* Hfr donor is *arom*⁻*met*⁻, whereas the *S. typhimurium* C5 recipient is *ile*⁻, requiring that a crossover take place between *arom* and *str* as well as between *rha* and *met* for the production of prototrophic hybrids. In addition, a crossover must occur between *met* and *inos* for the formation of *inos*⁺ recombinants.

Now, with the avirulence ($avir_1$) determinant as shown, some among those hybrids containing the region *ile*⁺*str*-r would be avirulent while others would remain unchanged, depending on whether the crossover took place to the right or left of this determinant. Similarly, some hybrids of the *inos*⁺ genotype would be avirulent while others would be unaffected, depending on the occurrence of a crossover to the right or left of

the position of $avir_2$ (Fig. 2). The crossover between *rha* and *met* is not associated with a change in virulence, since all the hybrids of the ile^+rha^+ class are still virulent. Thus, the depiction of at least two determinants controlling avirulence in the *S. abony* strain which are transferrable to *S. typhimurium* is compatible with the experimental findings. On this basis, the *complete* avirulence of the backcross hybrid which encompasses the regions of both the *str* and *inos* markers (Table 6) is interpreted as being due to the fact that it received both the $avir_1$ and $avir_2$ determinants of avirulence. Each determinant by itself would allow the phenotypic expression of partial avirulence, but both are presumably necessary for the expression of complete avirulence.

Although the significance of the spread in the partial avirulence pattern of the hybrids is not entirely clear in genetic terms, it may be that sites within the $avir_1$ determinant specify the quantitative aspects of avirulence as measured in terms of the LD_{50}. At the present time, it is not possible to state what the biochemical basis is likely to be for the alteration in the virulence of the C5 strain of *S. typhimurium*. It may be that the avirulence determinants in *S. abony* do not specify the synthesis of materials that the parent *S. typhimurium* strain has which are related to its mouse virulence, or possibly substances synthesized by the *S. abony* strain may mask the effects of those produced by the C5 strains. At any rate, the loss in virulence is not due to any nutritional requirement for various kinds of growth factors, as was found by Bacon, Burrows, and Yates (1951) in the case of *S. typhosa* and by Furness and Rowley (1956) for *S. typhimurium*. All of the hybrids examined in the present study were prototrophs except the $inos^+$ hybrids (Table 3), which still required histidine for growth. However, the requirement for histidine is unassociated with avirulence in view of the fact that parental recipient strains of C5 requiring histidine are virulent.

Recently, Falkow et al. (1963) found reductions in the virulence of hybrids of *Shigella flexneri* after recombination with *E. coli* K-12 donors. They used pretreated guinea pigs for which the *Shigella* strain was virulent, while the *E. coli* strain was avirulent, when challenge was made with a high dose level of bacteria. Their findings as to the location of the avirulence determinants of *E. coli* are generally compatible with the locations depicted by us for *S. abony*. The gross similarity of these results indicates that more extensive work on the genetic basis of bacterial virulence is needed with many different experimental systems to determine the possible significance of this concurrence.

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