INVESTIGATIONS ON THE GENETIC CONTROL OF VIRULENCE IN SALMONELLA TYPHIMURIUM

INVESTIGATIONS ON THE GENETIC CONTROL OF VIRULENCE IN SALMONELLA TYPHIMURIUM

by

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This thesis contains no material previously submitted for a degree in any University, either by the candidate or by any other person, except where due reference is made in the text of the thesis.

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SUMMARY

S.typhimurium strains may be characterized on the basis of their lethal capacity for mice. The introduction of a few bacteria of virulent strains into the peritoneum of mice is sufficient to kill a high proportion of animals. Avirulent strains require in excess of a million bacteria to produce the same effect. Since virulence is a stable property it may naturally be asked whether the expression of this property is under genetic control. If such a control were to be established, then the search for the biochemical differences between virulent and avirulent bacteria would be placed on a sound basis.

Advantage was therefore taken of the existence of genetic recombination mechanisms in Salmonella to investigate the problem.

In *S.typhimurium* strain C5, the loss of virulence of a leucine-requiring mutant was found to be unassociated with its growth requirement since the virulence of prototrophic transductants of the mutant was similar to that of the mutant. Immunologic studies of the mutant and its parental strain suggested that the differences in virulence may be due to a difference in some antigenic component other than the heat stable "O" somatic antigen complex of the two strains. In addition, the mutant was found to be unable

to multiply in the mouse peritoneum as rapidly as the parental virulent strain.

Attempts to develop a suitable recombination system between virulent and avirulent strains of S.typhimurium were unsuccessful. Subsequently an avirulent strain of S.abony was found to act as a chromosomal donor to the virulent strain C5 of S.typhimurium. An analysis of the hybrids arising from such crosses, showed that the segregation of two genetic loci - aviA and aviB was responsible for the expression of virulence intermediate between that of the donor and the recipient. It was further established by backcross experiments that both aviA and aviB were necessary for the expression of complete avirulence, characteristic of S.abony. On the basis of segregational analysis of the hybrids, aviA was mapped near the locus for streptomycin resistance/sensitivity while aviB was mapped adjacent to the locus for inositol utilization/non-utilization. The loci are separated by about 25 % of the bacterial chromosome.

Studies of the genetics of partial virulence between two S.typhimurium strains where the donor was partially virulent while the recipient was avirulent, were obscured because of anomalies associated with the analysis of linkage in hybrids.

The two-gene control of the difference in virulence

between S.abony and S.typhimurium strain C5 is discussed in relation to previous work along similar lines.

INTRODUCTION

Strains of S.typhimurium may be divided into those that are highly virulent for the mouse and those that are avirulent on the basis of the number of bacteria required to kill challenged groups of animals (see the introduction to Chapter 5). The virulence of a particular strain is generally found to be a stable characteristic since the effect is reproducible.

Previous studies have been concerned with analyses of the biochemical differences between virulent and avirulent strains of S.typhimurium (for example, see Maaløe, 1948; Jenkin, 1962). However, the recent development of genetic systems in S.typhimurium (see Chapter 2) afforded an opportunity to investigate the genetic control of virulence in this bacterial strain since this is a useful adjunct to the biochemical studies.

Chapter 1

NATURE AND DEFINITION OF VIRULENCE

One of the most striking aspects of the host-parasite relationship is the well known observation that host species specificity exists for microbial virulence. For example, under natural conditions the rat is insusceptible to infection with *Salmonella typhimurium* while the mouse is highly susceptible. Though the host animal species are closely related, their reaction to a single parasite is very different. The mouse on the contrary, is highly resistant to natural infections with *Salmonella paratyphi B*. Here even though *S.paratyphi B* is antigenically very similar to *S.typhimurium*, the mouse reacts quite differently to these bacteria. There is a whole spectrum of such specificity between animal hosts on the one hand and microbial agents on the other.

An experimental finding of similar importance is that different strains of the same species of bacteria (*S.typhimurium*) may produce widely different reactions in the same animal species (mouse) or vice versa (Furness and Rowley, 1956; Cowen, 1961). That is, disease is the outcome of the interactions between variations in the host or in the variations of the parasite. An additional variable is the environment. These complex interactions have to be

borne in mind in studying the hereditary basis for parasite or host variability. Due to the complexity of the variables involved, it is difficult to simulate under laboratory conditions, the natural situation. However, it is possible to study the hereditary basis for parasite or host variability by keeping reasonably constant any two variables and varying the third. This thesis will be concerned with investigations on the inheritance of parasite variability towards a particular host - the mouse. The host and the environment would be kept constant as far as possible.

Meaning of virulence.

Two words that are extensively used in studies of the host-parasite condition are virulence and pathogenicity. These two words present considerable semantic difficulty since they often describe the same condition (Wilson and Miles, 1955; Miles,1955; Rowley,1960; Raffel,1961). Obviously, in large measure, this is due to the problem of adequately considering the many attributes of disease. For the purpose of this thesis, the word virulence will be used and defined as follows. Virulence is the ability of a microorganism to cause disease in a particular host animal under specified conditions. That is, disease is encountered under natural conditions or when microorganisms are introduced artificially into the tissues of animals.

Virulence is here studied under the latter condition. If death of the animal is taken as the end result of disease then a clearcut assay is available to measure virulence. It is of course to be realized that death itself is the outcome of complex interactions between the host and the parasite and thus the expression of virulence in terms of death is an all-or-none phenomenon.

Chapter 2

LITERATURE REVIEW - GENETIC RECOMBINATION IN SALMONELLA

Two forms of gene transfer mechanisms have been discovered in Salmonella. One is bacteriophage-mediated transduction or conversion and the other is conjugation where cellular bridging between bacteria is obligatory. Although both forms are essentially alike, for practical purposes they may be distinguished on the basis of purely mechanical features of the process. The transfer of genetic material is unidirectional in that it takes place from donor to recipient bacteria. The transferred material (exogenote) may vary in size from very small fragments (in transduction or conversion) to nearly the whole haploid genome (in conjugation). DNA is implicated as the carrier of genetic specificity of the transferred material.

Transduction

Zinder and Lederberg (1952) discovered that cell-free filtrates from genetically marked strains of S.typhimurium were able to produce recombinants in other complementarily marked strains. They showed that bacteriophage particles were responsible for the activity of the cell-free filtrate. The unique feature of this process was that though the phage lysate was capable of effecting many different changes in recipient bacteria, any

single clone of recipients was only altered in respect of a single character. The phage bringing about this hereditary change is the temperate phage PLT-22.

The process involves firstly, the preparation of a phage lysate which may be obtained by infection of phage-sensitive donor cells with PLT-22. This leads to lysis and liberation of phage particles from all cells except from cells where lysogeny is established. If a complementarily marked recipient strain is infected with the phage lysate, then a proportion of recipient cells acquires some donor character. The frequency of transduction varies from 1 in 10^4 to 10^8 phage particles. It was initially thought that lysogenization of the recipient by phage was a prerequisite for the detection of transduction. But it is now known not to be necessary since virulent mutants of PLT-22 phage are capable of transduction with a high efficiency under suitable conditions (Zinder, 1959). The transducing phages act as vehicles for the carriage of fragments of genetic material from the donor to recipient bacteria. Though it is known in some instances that transducing phage particles are defective because of loss of phage functions, the crucial question of whether the same transducing particle transfers both bacterial genes and its own intact chromosome together is yet to be answered unequivocally (Luria, 1962).

Bacteriophage conversion

This process is similar to transduction except that in conversion every phage particle is capable of bringing about a hereditary change in infected recipient bacteria (Stocker, 1958). The converting phages are highly specific since in the best studied cases (for example, O-somatic antigen determinants in Salmonella) antigenic expression is solely controlled by the presence of these phages (Iseki and Sakai, 1953; Uetake et al., 1958; Uetake and Hagiwara, 1960). However, as in transduction, lysogenization is not a prerequisite for the expression of antigenic specificity. Infected recipient cells that are destined to lysis by the action of virulent mutants of converting phages can still express the new antigen within a few minutes after phage infection (Uetake et al., 1958).

Conjugation in strain K-12 of Escherichia coli

The first known form of genetic recombination in bacteria akin to sexual fertilization in higher organisms was discovered by Lederberg and Tatum (Lederberg, 1947). Mutants of E.coli K-12 were obtained by successive mutagenic treatment which were unable to synthesize essential growth substances (auxotrophs) whereas the original wild type strain could grow on a simple chemically defined medium (minimal medium). Multiple marked mutants which had complementary growth requirements, when plated

together on minimal medium, generated nutritionally independent (prototrophic) recombinants. An analysis of the linkage and segregation of different marker combinations showed that sexual recombination occurred in E.coli K-12. The necessity for cell contact (conjugation) for gene transfer was established by Davis (1950).

Since then a very exhaustive analysis of the process of sexual recombination in E.coli K-12 has been undertaken and the details have recently been documented (Jacob and Wollman,1961; Clark and Adelberg, 1962). It is intended that the salient features of the process will be discussed which form the basis of the extension of the phenomenon of conjugation to other enteric bacteria.

E.coli K-12 cells are phenotypically distinguished as either genetic donors (males) or genetic recipients (females) with respect to conjugation. Maleness is determined by the presence of a sex factor (F) which is a transmissible, genetic particle. Male bacteria are hence designated F+ and females F-. Only cells possessing F are capable of chromosome donation whilst F- bacteria are incapable of doing so. Donor bacteria are in fact, separable into 3 types: F+, Hfr (high frequency of recombination) and F' (F-prime). The sex factor in F+ bacteria exists autonomously since such bacteria treated with acridine orange lose F and become F-. Moreover, the sex

factor in F+ bacteria is transferred to F- cells at very high frequency and independently of chromosome transfer. The latter transfer is of very low frequency (less than 0.1% for a given marker). It is suspected that chromosome transfer by F+ cells is partly due to Hfr cells in the population since Hfr mutants can be isolated from F+ bacteria. The frequency of transfer of chromosomal markers by Hfr clones may be as high as 100 % for a lead marker and different Hfr strains transfer their chromosome in a characteristic linear sequence. Each Hfr has a different transfer orientation. It has a leading point or origin that is followed by a definite sequence of markers which may be different for different Hfrs. The transfer of the whole chromosome takes 107 minutes at 37° C, but the speed of transfer is constant over the entire period. This means that distances between markers may be easily expressed in terms of the time difference between markers. During transfer spontaneous breaks in the chromosome occur and this has been shown to have a certain probability per unit of length. Consequently, the further away a marker is from the origin, the lower is its probability of being transferred. Experimentally this is attested to by the finding of a gradient of recombination frequencies such that proximal markers are transferred at very much higher frequency than distal

markers. F in Hfr cells is stably attached to the chromosome and is the last marker to be transferred. Its chromosomal stability is inferred because of its resistance to disinfection with acridine orange though it occassionally breaks away. When this happens the bacteria revert to the F+ type.

Hfr strains transfer the chromosome in a linear sequence. But different Hfr donors may have different transfer sequences. Their relationship was understood when the chromosome of F+ bacteria (from which the Hfr mutants arose) was conceived to be circular. Attachment of F to the chromosome leads to chromosome rupture and attachment can occur anywhere along the chromosome. The break occurs adjacent to the F attachment site. During transfer the chromosome is linear and it proceeds from the origin (opposite end to the F attachment site) towards the sex factor end. Under these conditions F is transferred as a chromosomal marker which is at the end of the transfer sequence.

In rare circumstances F' cells arise from Hfr populations when F breaks away from the chromosome carrying with it chromosomal fragments and returns to its autonomous state. In this condition F' cells are identical in behaviour to F+ cells except that the former harbour F intimately associated with chromosomal

fragments (merogenotes) and they transmit the merogenote at very high frequency. The merogenote is derived from the chromosomal location to which F was attached. It is visualized to have a "memory" for the homologous chromosomal region since it can synapse with that region and generate Hfr cells identical in their gene transfer behaviour to the original Hfr population from which the F' strain arose. Thus F' bacteria are capable of transferring the merogenote and the chromosome independently and at very high frequency.

Extent of conjugal recombination in Escherichia coli strains other than strain K-12

Having established the sexual recombination system in E.coli K-12 it was natural to ask whether strain K-12 was unique or whether other strains of E.coli were also influenced by a similar breeding system. Using the streptomycin-resistant prototroph (SRP) selection technique, Lederberg tested 2,000 non-K-12 strains of E.coli (Lederberg 1951; Lederberg et al., 1952). By this method, prototrophic, streptomycin - resistant recombinants were sought for by crossing known K-12 donor or recipient tester stocks that were auxotrophic and streptomycin-resistant with the unknown which was prototrophic and streptomycin sensitive. It was found that about 4 % were fertile with the testers. However, this low number

of compatible species was not surprising since they were studied at a time when the complexity of the mating type system in E.coli K-12 was not clearly understood. More recently, mating compatibility has been re-examined by Ørskov and Ørskov (1961). They also used the SRP method but the tester strains were known Hfr, F+ or F- derivatives of strain K-12 and 2 other strains. The 199 E.coli strains they screened had characterisable antigenic type specificities. About 35 % were found to be fertile. One reason postulated by the Ørskovs for the increased number of fertile species was that the envelope antigens in E.coli might interfere with the initial pairing necessary for conjugation. This was based on the fact that most of the strains used by Lederberg were fresh isolates from pathological cases and therefore likely to have well developed O and K antigens, whereas the strains used by the Ørskovs were mostly old laboratory stocks and hence could have had poorly developed surface antigens. Though this postulation is very attractive, no experimental evidence exists for it (Ørskov and Ørskov, 1961).

The SRP method has definite limitations. The most serious is in its use to detect donor strains. In the survey of Ørskov and Ørskov (1961) one of the F- tester strains was histidine and isoleucine dependent. In order for recombinants to be formed, the donor must transfer

both the respective prototrophic alleles to the same recipient cell. Since the histidine and isoleucine loci are quite far apart on the E.coli K-12 chromosome (Jacob and Wollman, 1961) it is unlikely that detectable numbers of recipient cells would have received both the alleles. A less serious disadvantage accrues from the use of Hfr tester strains whose gene transfer orientation is not known, as for example in the study of Ørskov and Ørskov (1961). To detect recipient strains, the streptomycin-resistant allele must be transferred into them. If the Hfr transfers this allele as a distal marker then the frequency of appearance of the allele in recipients will be drastically reduced. Therefore, it might have been preferable to use the Hfr strains which transfer the streptomycin-resistant allele as a lead marker. Though the main advantage of the HRP technique is the rapidity with which large numbers of strains can be examined for fertility, the use of many different confirmatory tests to detect fertility would obviously increase the scope of such studies, (for example, Mäkelä et al., 1962).

Sexual recombination between Escherichia and Salmonella

The first successful mating between bacteria of different genera was achieved by Luria and Burrous (1957). They showed that many antigenic types of Shigella were capable of acting as recipients in genetic crosses with

E.coli K-12 Hfr or F+ donors. The Shigellas behaved as F- equivalents in the fertility system of E.coli K-12. Using zygotic induction of transferred prophage as a measure of conjugal capacity it was found that Shigella strains were equally as capable of conjugating with E.coli K-12 donors as were homologous K-12 crosses. In contrast, the frequency of recombination was 100 - 1000 times lower in the Shigella x E.coli crosses than in E.coli x E.coli crosses. In addition some markers from E.coli were never detected in the hybrids, though in some instances fairly extensive transfer was observed. To what extent this lack of transfer was due to the choice of K-12 Hfr strains is not known. It will be expected that the detection of distal markers in hybrids selected for the reception of early markers by a particular Hfr will be seriously affected by spontaneous breaks in the transferred chromosome. However, Luria and Burrous (1957) have suggested an incomplete homology between the genomes of the two heterologous bacteria as a reason for the disparity.

Isolation of F- Salmonella

In 1958, Baron and his collaborators reported recombination between species of Salmonella and E.coli K-12 donors (Baron et al., 1958). Though the frequency of recombination of the selected marker lac+ (capacity to

utilize lactose) was low (10^{-8} per donor cell) in crosses with strain TM-9 of S.typhimurium, a streptomycin-resistant mutant - TM-9Str-r-2 was isolated which showed a much higher frequency (Baron et al., 1959a). Strain TM-9Str-r-2 recombined with an E.coli K-12 Hfr at 10^{-4} when lac+ was the initially selected marker. In addition, the streptomycin-resistant derivative of strain TM-9 was able to cross with a F+ E.coli K-12 strain at 10^{-6} whereas TM-9 failed to recombine with it. That the increased recipient capacity of TM-9Str-r-2 was only fortuitously associated with the acquisition of streptomycin-resistance was established when a large number of individual str-r clones of strain TM-9 and other S.typhimurium strains showed recombination frequencies characteristic of strain TM-9 (Baron et al., 1959a). Using the high frequency genetic recipient it was then possible to study the extent of chromosomal transfer from an E.coli K-12 Hfr into S.typhimurium TM-9Str-r-2. It was found that in addition to the transfer of the lead marker (lac+) other markers were capable of being transferred at low frequency.

In a subsequent study Baron et al.,(1959b) noticed that a very large number of Salmonella species were incapable of acting as recipients of genetic material from an E.coli donor. In order to explain the existence of the high frequency recipient S.typhimurium strain TM-9Str-r-2,

previous to mating experience, Baron postulated that Salmonella cells are initially sterile (Fo) with E.coli donors. However rare mutational changes occur amongst these cells which give rise to F- bacteria. Such bacteria are therefore assumed to be the ones which recombine with Hfr E.coli at low frequency. It was proposed therefore, that an initially obtained recombinant from an E.coli x Salmonella cross should recombine at a very much higher frequency on subsequent mating with E.coli. The hypothesis was tested with a genetically marked S.typhosa 643 Vi-positive strain. An initially obtained lac+ hybrid was backcrossed with the same E.coli Hfr, selection being made for the arabinose-utilization marker (ara+), which is close to lac+. The result was that the lac+ hybrid recombined at a relatively higher frequency than the unmated S.typhosa for the ara+ marker (Baron et al., 1959b). Numerical increases in frequency were determined when an initial lac- hybrid (obtained from a cross: E.coli Hfr lac- ara+ x S.typhosa 643 Vi-positive lac+ ara-) was backcrossed with a lac+ Hfr E.coli. The frequency for lac+ was about 1×10^{-4} per donor cell. This was reminiscent of the recombination frequency characteristic of the S.typhimurium strain TM-98tr-r-2 which was isolated prior to mating experience.

Miyake and Demerec (1959) working with another

S.typhimurium strain, LT-7, discovered that it recombined with an E.coli K-12 donor that was similar to that used by Baron et al., (1959a,b). The frequency of recombination was low (10^{-6} of plated bacteria) and it only occurred when the LT-7 strain possessed a mutator gene. The function of the mutator was to non-specifically increase 10-100 fold the spontaneous mutability of other genes. The hybrids were found to have received in addition to the first marker (lac+) transferred by the E.coli Hfr, other adjacent loci. When these hybrids were backcrossed with the same donor, an increase (10-100 fold) in the recombination frequency was observed over the initial frequency (Miyake, 1959). Miyake (1959) considered two possibilities for this increase : (1) that it was due to the presence of chromosomal or cytoplasmic material in the hybrids derived from the donor parent; (2) that mutation of a fertility factor by the mutator gene in strain LT-7 was responsible. Experimentally, the alternatives were tested by the technique of replica plating. He was able to isolate about 1 % fertile clones from the mutator carrying LT-7 strain which had no previous sexual contact with E.coli. This result supported his second proposition. The spontaneously occurring fertile clones were not peculiarly receptive to one donor only but displayed a high frequency recipient

capacity to a number of E.coli K-12 donors (Miyake,1959). From these findings it was suggested that the mutator-bearing LT-7 strain of S.typhimurium was a mixture of fertile and infertile cells and that only the former recombined with E.coli donors. Since recombination was also observed between an E.coli F+ donor and the fertile Salmonella, although at very low frequency, Miyake (1959) proposed that the fertile recipient was equivalent to F- but that the infertile type was F+. The mutator gene was said to increase the frequency of mutation from F+→F-. It is difficult to see how the infertile clones are equivalent to F+ since if they were F+ in the sense of donor in E.coli K-12 then F+ Salmonella should cross with F- Salmonella. In fact Miyake (1962) failed to obtain recombination in this case. It would be better to recognise the infertile clones as Fo (Baron et al., 1959b).

The question of the increase in the backcross recombination frequency of hybrids over the initial frequency has been examined by Johnson et al., (1963) with the emphasis being on the influence of the initially transferred E.coli donor material. It was found in studies with S.typhosa 643 Str-r (non-Vi and streptomycin-resistant) as recipient and a number of E.coli K-12 donors that the increased fertility of the hybrids was due to the presence of genetic material from the proximal

region of the initial Hfr. Replica plating experiments of the kind performed by Miyake (1959) were also repeated by Johnson et al., (1963) in their system. Contrary to Miyake's finding in S.typhimurium LT-7, it was observed that S.typhosa 643 Str-r was relatively homogeneous with respect to initial fertility.

Infectious transfer of F into S.typhimurium

The ability of the E.coli K-12 sex factor (F) to be transferred into F- bacteria has been exploited by Zinder (1960b) in his work with S.typhimurium strain LT-2. When cultures of F+ E.coli and LT-2 are cross-streaked on eosin methylene blue medium (without sugar) the resulting growth produces a red staining reaction at the junction of the streaks. From these red areas Zinder was able to isolate F+ clones of S.typhimurium LT-2 which were previously infertile. The possession of the sex factor was confirmed by the reverse transfer of F into E.coli F- cells. Preliminary experiments showed that the recombination and linkage pattern of F+ x F- LT-2 crosses were similar to their counterparts in E.coli K-12.

Extent of sexual compatibility promoted by the E.coli F' sex factor in Salmonella

So far the emphasis has been on E.coli x Salmonella recombination mediated by the wild type sex factor of the K-12 strain. However, the existence of E.coli K-12

donors which transfer at very high frequency the sex factor associated with a chromosomal fragment (F') has afforded an analysis of the range of sexual competence in the Salmonella group. The merogenote associated with F' is lac+ in this case and since most naturally occurring species of Salmonella are lac-, a readily available selective device is present to study the fertility of Salmonella. In an initial survey, Ørskov et al., (1961) reported that about 50 % of 86 Salmonella species representing all the O groups of the Kauffmann-White scheme were fertile with the F' lac donor of E.coli K-12. In a more detailed analysis of mating compatibility, Mäkelä et al., (1962) have studied the fertility patterns of enteric bacteria using a closely related F' donor to the one used by Ørskov et al., (1961). By a number of criteria the transfer of F' has been successful not only from E.coli into other enteric bacteria but also in various combinations within these bacteria. Large differences were found in the ability of strains to receive F' but characteristically homologous combinations gave the highest degree of transfer and fertility. Though promiscuity within the Enterobacteriaceae is thus established, the more important and arduous task of studying in detail the genetics of as many single species as possible is awaited. Along these lines Mäkelä (1963a) has

isolated several Hfr mutants of S.abony which were initially infected with the wild type sex factor of E.coli K-12 (Mäkelä et al., 1962). Others that could be profitably explored are S.miami, S.muenster and S.senftenberg which were easily infected with F or F' (Mäkelä et al., 1962). Such studies could give valuable information on whether the E.coli K-12 breeding system is of universal import in enteric bacteria or whether other as yet undescribed patterns of gene transfer are operative.

Genetic stability of hybrids of E.coli x Salmonella crosses

In recombination experiments between an E.coli K-12 donor and S.typhosa Vi-positive recipient, it was observed that the transfer of large segments of donor chromosomal material led to its non-integration in recipient bacteria, (Baron et al.,1960). They were detected because of the inability of these clones to be purified either on selective or non-selective media. They continually segregated haploid segregants characteristic of the S.typhosa parent. A genetic analysis of the markers carried by these unstable clones led to the conclusion that they were partial diploid heterozygotes (Baron et al., 1960). The diploid region extended over about one-third of the chromosome from lac+ which is the lead marker of the K-12 donor to the marker for xylose

utilization. By repeated reisolations on the initial selective medium it was possible to obtain relatively stable diploid hybrids. A detailed genetic analysis of the segregants of such hybrids and also diploids arising from S.typhimurium TM-9Str-r-2 as recipients, was performed by Falkow et al.,(1962) to determine which alleles of the E.coli parent were integrated. It was found that the relatively stable diploid hybrids were only diploid for a very small region in the middle of the transferred chromosomal segment. Two main types of haploid segregants were obtained. In one, the entire coli segment which was about a third of the chromosome was integrated in the Salmonella hybrids. In the other, recombination occurred between the coli material and its homologue in Salmonella which led to the integration of all the E.coli alleles except the small middle region which was still Salmonella material. It appeared, therefore, that the transfer of about 12 % of the coli chromosome into Salmonella led to integration but material in excess of this amount had difficulty in being integrated though it could remain non-integrated in recipient bacteria (Falkow et al., 1962).

High frequency of recombination (Hfr) donors in Salmonella

The extensive examination of the E.coli K-12 chromosome was only possible when Hfr donors were available to

map the many loci now known in that strain (Jacob and Wollman, 1961). Therefore, it was natural to look for Hfr Salmonella donors so that similar studies could be initiated in the Salmonellae. Zinder (1960b) obtained the first reported Hfr in S.typhimurium LT-2 after strain LT-2 was converted to F+. Miyake (1962) isolated an Hfr in S.typhimurium LT-7 using the mutator carrying LT-7 F- recipient and the E.coli K-12 Hfr P4X-6. The sex factor in the K-12 Hfr is located adjacent to lac+ and lac+ is the last marker transferred by the E.coli donor. Hence Miyake (1962) selected for lac+ recombinants in crosses with the E.coli K-12 Hfr and the S.typhimurium recipient. He found that lac+ hybrids so obtained had also concurrently become Hfr donors. One such S.typhimurium Hfr was tested for its donor ability to non-mutator LT-7 strains. Positive results were obtained, indicating that even though non-mutator strains were sterile with E.coli K-12 donors they were compatible with a S.typhimurium Hfr whose chromosomally attached F was derived from a K-12 Hfr. Baron et al., (1963) have also isolated an Hfr from S.typhimurium strain TM-9Str-r -2 by a similar process to that of Miyake (1962) and using the same E.coli K-12 Hfr. Using the above mentioned Hfr strains of S.typhimurium, Baron et al.,(1963) have performed genetic recombination with either S.typhimurium

or S.typhosa as recipient. The Salmonella Hfr strains reacted genetically as did E.coli K-12 Hfr donors and in particular Miyake's and Baron's Hfr strains had gene transfer orientations identical to the E.coli Hfr from which they were derived.

Mäkelä (1963a) has isolated 7 Hfr derivatives after ultra-violet irradiation of an F+ culture of S.abony which had received F from E.coli K-12. The donor strains showed different gene transfer orientations. One of them was like a typical E.coli K-12 Hfr in that F was non-infectious. The others resembled Richter's E.coli K-12 Hfr in which F alternates between the infectious and a specific chromosomal attachment site (Richter,1961). When F was infectively transferred from the atypical S.abony Hfr strains to F- bacteria the converted cells became F+, not Hfr. Upon removal of F from the Hfr it reacted as F- which on subsequent reinfection with F was converted to an Hfr of the original type. In other words, they had a specific sex factor affinity locus on the chromosome.

Colicinogeny-mediated conjugal recombination in Salmonella

Colicinogenic factors I and E1 have been found to promote genetic recombination in S.typhimurium strain LT-2 (Ozeki and Howarth, 1961). Freshly prepared colicinogenic I donors recombined with non-colicinogenic LT-2

strains at the low frequency of 10^{-8} of plated bacteria. But the frequency was increased 100 fold when the donor population consisted of a mixture of factors I and E1 cells. A characteristic of gene transfer in this system was that all tested donor alleles were transferred at the same frequency. In addition, it has been shown that this form of recombination is not promoted by the E.coli sex factor (Ozeki et al., 1962). The general features of this process have been likened to sexual recombination in E.coli K-12 (Ozeki and Howarth, 1961). For example, it was visualised that recombination between colicinogenic donor and non-colicinogenic recipient bacteria was equivalent to F+ x F- E.coli K-12 crosses. Factor E1 was thought to lead to breakage of the chromosome which was equivalent to the formation of Hfr mutants in F+ populations of E.coli. This was postulated to explain the increased frequency of recombination in colicinogeny-mediated recombination (Ozeki and Howarth, 1961). A puzzling observation was that only freshly prepared factor I cells transfer colicinogeny and chromosomal genes. It has been hypothesized that in old stock cultures the I factor is chromosomally integrated (Smith and Stocker, 1962). If this integration is equivalent to the Hfr state in E.coli then the lack of transfer by old colicinogenic cultures is unexplained. It would appear

therefore, that colicinogeny-mediated recombination may differ fundamentally from sexual recombination in E.coli K-12. Meynell (1962) has studied a similar situation in S.enteritidis. Though in S.enteritidis factors I and E1 promote similar kinds of events as in S.typhimurium LT-2, chromosome transfer is very low (10^{-9} of recipient bacteria) in crosses between colicinogenic S.enteritidis donors and S.typhimurium recipients. Recombination was not detected using either S.enteritidis or S.typhimurium as donors and S.typhosa as recipient. Lack of recombination was shown not to be due to lack of conjugation since the colicinogenic factors themselves were transferred at very high frequency.

Chromosomal homology between E.coli and Salmonella

Zinder (1960b) first observed in recombination experiments using an E.coli K-12 Hfr and S.typhimurium LT-7 that the gene order for about 8 % of the Salmonella chromosome was identical to E.coli K-12. Using the same system but by analysing a larger number of loci within that same region, Miyake (1962) confirmed Zinder's finding. Falkow et al., (1962) have used interrupted mating experiments to determine not only the gene order but the distance between loci in time units in genetic crosses using the same E.coli K-12 Hfr but with S.typhimurium TM-9Str-r-2 and S.typhosa 643 as recipients. An examination of about

1/3 of the bacterial chromosome showed not only an identical gene order but also a similarity in time units between markers when the E.coli K-12 map (Jacob and Wollman, 1961) was compared with the Salmonella map. An unexplained observation in the behaviour of the same K-12 Hfr in crosses with E.coli F- and Salmonella recipients was the delay in chromosome transfer. The lead marker lac+ was transferred into E.coli in about 3 minutes (Jacob and Wollman, 1961) but into Salmonella in 12 minutes (Falkow et al., 1962). Though these studies point strongly to the similarity in linkage structure between E.coli and Salmonella, interrupted mating experiments using as many different Hfr donors and recipients as possible, are necessary before a detailed comparison could be made.

In colicinogeny-mediated recombination, Smith and Stocker (1962) have found that the gene order throughout the entire chromosome in S.typhimurium LT-2 is similar to E.coli K-12. Moreover, their linkage data was interpreted as indicating of a circular chromosome as in E.coli K-12. Because of the absence of donors with orientated transfer sequences in this system it is not possible to compare directly the distance between different loci between S.typhimurium LT-2 and E.coli K-12. Preliminary studies of S.abony using 6 different Hfr

donors has also pointed to a similarity in gene order between S.abony and E.coli (Mäkelä, 1963a). The S.abony results were again compatible with a circular chromosome.

In spite of the overall homology of chromosome structure, there is evidence of incomplete homology at the fine structure level. For example, Zinder (1960a) observed that the transduction of E.coli genes into Salmonella and vice versa by phage PLT-22 was seriously impaired though more extensive chromosome transfer via conjugation occurred quite readily (Zinder, 1960b). He suggested that this was due to the non-identity of micro-structure since obviously identity would be necessary for synapsis over short distances before recombinant formation. Secondly, the frequency of recombination in crosses between E.coli and Salmonella was very much lower than comparable frequencies with E.coli x E.coli crosses (Falkow et al., 1962). In addition, in crosses between S.typhimurium Hfr as donor and S.typhosa as recipient there was little linked transfer though recombinants with the selected marker were obtained easily when selection was made for widely separated donor alleles (Baron et al., 1963). Thirdly, extensive transfer of genetic material from E.coli into Salmonella led to the formation of segregating partial heterozygotes (Baron et al., 1960; Falkow et al., 1962). Fourthly, DNA hybridization

experiments indicated that although the overall base composition of E.coli and Salmonella DNA was similar, no molecular hybrids were obtained with their respective DNA preparations. However, partial heterozygotes possessing varying amounts of E.coli material did form DNA hybrids with E.coli DNA commensurate with the extent of integrated coli material in the genetic hybrids (Falkow et al., 1962). Inhomology has therefore, been interpreted as being due to imperfect pairing at the level of DNA molecules which consequently leads to low recombinational events (Falkow et al., 1962). Presumably the pairing difficulties are due to the base sequence differentials in donor and recipient DNA molecules.

Chapter 3

MATERIALS AND METHODS

1. Bacterial and Bacteriophage strains

Bacterial strains and gene symbols

The list of strains used is shown in Table 1. All the Salmonella strains were smooth in colonial appearance whereas the E.coli strains were rough. The pertinent genetic markers and the derivation of each strain is also shown. Grateful acknowledgement is made to the numerous investigators who gave many of the initial strains mentioned in Table 1. The symbols used to signify genetic markers are explained in Table 2. It is based as far as possible on the symbolism proposed in Microbial Genetics Bulletin (1963).

Maintenance of strains

When received the strains were plated out on nutrient agar and single colonies were tested for their markers. They were then freeze dried from broth on a freeze drying apparatus. The ampoules were kept at room temperature. When necessary, an ampoule was opened and the resuspended culture from the ampoule was plated out on nutrient agar. Single colonies from the agar medium, that had been tested for their markers, were planted on nutrient agar slopes. These formed the working cultures

TABLE 1 LIST OF BACTERIAL STRAINS USED

Strain designation	From strain	Genetic markers	Derivation	Selected references
E.coli				
W2586[a]	K-12	F-, colicine indicator	-	-
CL136(E1)[b]	K-12	F+, his-,str-r, (E1)	From K-12 by mixed culture with *E.coli* K-30(E1)	Ozeki et al.,(1962)
W1895[a]	K-12	Hfr,met-,lac+	-	Baron et al.,(1959a)
P4X-6[a]	K-12	Hfr,met-,lac+	-	Miyake (1962)
S.abony				
SW1444[c]	74	Hfr,met-,arom-,ara+, rha+,xyl+,inl+,mtl+, srl+,gal+,lac-,mal+, str-r, H1-b,H2-e,n,x	Identical to SW1391 (see ref.). U-V irradiation and selection from *S.abony* F+[§]	Mäkelä (1963a)
S.typhi-murium				
cys D-36(I)[b]	LT-2	cys-, (I)	From cys D-36 by mixed culture with *S.sonnei* P9 (I,E2)	Ozeki et al.,(1962)

TABLE 1 (CONT.)

Strain designation	From strain	Genetic markers	Derivation	Selected references
cys D-36(B)[b]	LT-2	cys-,(B)	From cys D-36 by mixed culture with *E.coli* K77(B)	Ozeki et al.,(1962)
cys D-36(E1)[b]	LT-2	cys-,(E1)	From cys D-36 by mixed culture with CL136(E1)	Ozeki et al.,(1962)
cys C-7(K)[b]	LT-2	cys-,(K),str-r	From cys C-7 Str-r by mixed culture with *E.coli* K49(K)	Ozeki et al.,(1962)
LT-2 wild type[d]	LT-2	prototrophic, lac-	-	Furness and Rowley (1956)
SR305[e]	LT-2	Hfr,his-,rha+,mal+, ara+,gal-,xyl+,inl+, mtl+,srl+,lac-,dul-, str-s	U-V irradiation and selection from LT-2 F+[f]	Zinder (1960b)
SR305 Gal+	LT-2	As SR305 except gal+	From SR305 by selection*	-
37[a]	37	nic-,ara+,gal+,rha+, mal+,xyl+,inl-,mtl+, srl+,lac-,dul-,str-s	-	-

TABLE 1 (CONT.)

Strain designation	From strain	Genetic markers	Derivation	Selected references
37(I)	37	As 37 except (I)	From 37 by mixed culture with cys D-36 (I)*	-
37(B1)	37	As 37 except (B1)	From 37 by mixed culture with CL136 (B1)*	-
62G[a]	62G	As 37	-	-
SW1292[a]	TM-9 Str-r-2	pro-,leu-,ara-,gal-, rha-,mal-,xyl-,inl+, str-r	Originally isolated as str-r mutant from TM-9	Falkow et al.,(1962)
15-11[a]	TM-9 Str-r-2	Hfr,pro-,arg-,leu-, ura-,rha+,inl+,lac+, str-r	Isolated as Hfr from cross: TM-9 Str-r-2 x P4X-6	Baron et al.,(1963)
175(I)[f]	LT-7	pro-,arg-,ile-,try-, thr-,gal-,(I),str-r	U-V irradiation and selection from LT-7	-
Br1[g]	Br1	prototrophic, lac-	-	Jensen (1929)

TABLE 1 (CONT.)

Strain designation	From strain	Genetic markers	Derivation	Selected references
M206 wild type[d]	M206	prototrophic,inl-, rha+,ara+,gal+,mal+, xyl+,lac-,str-s	From Br1 by cultivation at increasing growth- temperature (Jensen,1929)	Furness and Rowley (1956)
M206 Ser-	M206	As M206 wild type except ser-	From M206 wild type by U-V irradiation and penicillin selection*	-
C5 wild type[d]	C5	prototrophic,inl-, rha-,ara+,gal+,mal+, xyl+,mtl+,srl+,lac-, dul-,str-s,H1-i, H2-1,2	-	Furness and Rowley (1956)
C5 His-	C5	As C5 wild type except his-	From C5 wild type by U-V irradiation and selection*	-
C5 His-(I)	C5	As C5 His- except (I)	From C5 His- by mixed culture with cys D-36(I)*	-

TABLE 1 (CONT.)

Strain designation	From strain	Genetic markers	Derivation	Selected references
C5 His-(Z1)	C5	As C5 His- except (Z1)	From C5 His- by mixed culture with CL136(Z1)*	-
C5 His-Ara-	C5	As C5 His- except ara-	From C5 His- by EMS treatment and penicillin selection*	-
C5 His+Ara-Str-r	C5	As C5 His-Ara- except his+,str-r	From cross: SW1444 x C5 His-Ara-	-
C5 Ile-	C5	As C5 wild type except ile-	From C5 wild type by EMS treatment*	-
C5 Leu-	C5	As C5 wild type except leu-	From C5 wild type by U-V irradiation and penicillin selection*	-
C5 Val-	C5	As C5 wild type except val-	From C5 wild type by EMS treatment*	-
C5 Met-	C5	As C5 wild type except met -	From C5 wild type by U-V irradiation and penicillin selection*	-

TABLE 1 (CONT.)

Strain designation	From strain	Genetic markers	Derivation	Selected references
C5 Try-	C5	As C5 wild type except try-	From C5 wild type by U-V irradiation and penicillin selection*	-
C5 Cys-	C5	As C5 wild type except cys-	From C5 wild type by EMS treatment*	-
C5 Arg-	C5	As C5 wild type except arg-	From C5 wild type by EMS treatment*	-
C5 Ade-	C5	As C5 wild type except ade-	From C5 wild type by U-V irradiation and penicillin selection*	-
Q1[h]	Q1	Non-lysogenic. Phage propagating strain	-	Boyd and Bidwell (1957)

*See Materials and Methods'; [b]The sex factor (F) was derived from E.coli K-12
Strains designated"a - h" were kindly given by : [a]Dr.L.S.Baron; [b]Dr.B.A.D.Stocker;
[c]Dr.P.H.Mäkelä; [d]Prof. D.Rowley;
[e]Dr.N.D.Zinder; [f]Dr. S.Falkow;
[g]Prof.F.Kauffmann; [h]Dr.N.Atkinson.

TABLE 2 SYMBOLS USED TO DENOTE GENETIC MARKERS

(a) Growth factor requirements

Symbol	Meaning	Symbol	Meaning
ade	adenine	nic	nicotinic acid
arg	arginine	pro	proline
arom	aromatic amino acids (phenylalanine + tyrosine)	ser	serine
cys	cystine	thr	threonine
his	histidine	try	tryptophane
ile	isoleucine	ura	uracil
leu	leucine	val	valine
met	methionine		

\+ = requirement; - = non-requirement

(b) Carbohydrate compounds as energy sources

Symbol	Meaning	Symbol	Meaning
ara	arabinose	mal	maltose
dul	dulcitol	mtl	mannitol
gal	galactose	rha	rhamnose
inl	inositol	srl	sorbitol
lac	lactose	xyl	xylose

\+ = utilized as carbon source or fermented; - = not utilized or fermented

TABLE 2 (CONT.)

(c) Colicinogenic determinants

Symbol	Meaning
(B)	colicinogenic for colicine B
(E1)	colicinogenic for colicine E1
(E2)	colicinogenic for colicine E2
(I)	colicinogenic for colicine I
(K)	colicinogenic for colicine K

(d) Antibacterial chemical

Symbol	Meaning
str	streptomycin

r = resistance; s = sensitivity

(e) Flagella serotypes

Symbol	Meaning
H1	phase one antigen
H2	phase two antigen

(f) Fertility characteristics

Symbol	Meaning
F	sex (fertility) factor
Hfr	high frequency of recombination strain

\+ = strain possessing F; - = strain not possessing F

and they were maintained either at 4° C or at room temperature. When kept at 4° C, they were sub-cultured onto fresh slopes once every 6-9 months. Periodically all working cultures were tested for their markers.

Testing for markers

S.typhimurium strains were identified by slide agglutination using diluted specific antiserum. A small amount of growth from a single colony was emulsified with a loopful of serum on a slide. Agglutination was indicated by rapid clumping of bacteria.

Nutritional requirements were confirmed by the presence or absence of growth when a washed culture was spread, or a small amount of growth from a single colony was streaked out, on LM or minimal agar in the presence or absence of growth factors. Alternatively, a lawn plate on LM agar was sometimes used and small filter paper discs dipped in growth factor solutions were then placed on the lawn. Double or multiple requirements were confirmed by studying various combinations of additions to synthetic medium.

Sugar markers were tested by spreading washed cultures on minimal medium supplemented with a particular carbohydrate as the carbon source plus the strain's growth requirements. Alternatively, they were checked in liquid indicator medium. Response to streptomycin was tested on nutrient agar plus streptomycin. Colicinogeny

was tested by the production of colicine against the indicator strain W2586 (see below). The fertility type of strains was confirmed by suitable crosses.

Bacteriophage strains

Temperate phage PLT-22 used in transduction experiments was originally from J. Lederberg (Zinder and Lederberg, 1952). It was kindly given by Mr. A.H.Rogers.

A number of temperate and virulent phages were used to confirm the derivation of S.typhimurium C5 Leu- from C5 wild type (Table 4: Chapter 4). All the phages were kindly donated by Dr. N. Atkinson. The phages PB. Jersey, PB. BAOR, PB.3b, PB.01, PB.02 and PB.03, were originally from Dr. E.S. Anderson, Enteric Laboratory, Central Public Health Laboratory, Colindale, London, England. The phages TMA3, TMA4, and TMA1a were originally from Brig. J.S.K. Boyd, Wellcome Research Institute, London, England. The phages B43h/49 and D39h/41 were the phages described by Atkinson and Bullas (1957). The phages TM7/Q1, SD7/Q1, S162/Q1 and H3/Q1 were phages described by Rogers (1960). All phages were propagated on S.typhimurium Q1 (non-lysogenic strain) in liquid medium by the technique described by Adams (1959). Chloroform was added to all the phage stocks to kill residual organisms. Dissolved chloroform was removed before use by bubbling sterile air through the stock solution.

All phage stocks were kept at 4° C.

2. Media

Saline - 0.85% NaCl in distilled water

Media for the routine culture of bacteria

Nutrient Broth No.2 (Oxoid Ltd., London E.C.4, England Code No. CM67) and Penassay Broth (Difco Laboratories, Detroit, Michigan, U.S.A.) were used as liquid media and in the thesis they will be referred to as broth. Nutrient agar (dehydrated Blood Agar Base; Oxoid Ltd., London E.C.4, Code No.CM55) and meat extract agar (Difco) were used as solid media and they will be referred to as nutrient agar. These media were made up as directed.

Soft agar overlay medium

It consisted of meat-extract agar (Difco) made up with one-half the amount used for making solid nutrient medium per litre of water.

Minimal medium

Two types of media were used. They differed in the relative concentration of the constituent salts though both types served the same purpose.

(i) Minimal medium of Lederberg (1950). The salts were dissolved in water at 10 times the required concentration and autoclaved. The 10 x concentrated

salt solution consisted of :-

$K_2 HPO_4$	-	70 gm.
KH_2PO_4	-	20 "
Sod.Citrate. $5H_2O$	-	5 "
Mg. SO_4. $7H_2O$	-	1 "
$(NH_4)_2 SO_4$	-	10 "

dissolved in 1 litre of distilled water. The pH was adjusted to 7.0. For minimal liquid medium the concentrate was diluted 1/10 and a sterile 20% glucose solution was added to give 0.2 % glucose. For minimal agar medium the exact procedure was followed except the concentrated salt solution and glucose were diluted into a 1.5 % solution of Bacto-agar (Difco) which had previously been dissolved and sterilized by autoclaving. This medium will be referred to as LM medium in the text and at all other times minimal medium will refer to the preparation described below.

(ii) The minimal medium was identical to the formula prescribed in "Selected methods in bacterial genetics" (1963) except that citrate was omitted since Salmonellae could utilize it as a carbon source. The 10 times concentrated salt solution was composed of:-

K_2HPO_4	-	105.0 gm.
KH_2PO_4	-	45.0 "
$Mg\ SO_4 \cdot 7H_2O$	-	0.5 "
$(NH_4)_2\ SO_4$	-	10.0 "

dissolved in 1 litre of distilled water and was autoclave sterilized. For minimal agar medium, 80 ml. of the concentrate and 320 ml. of sterile distilled water were added to a separately autoclaved Noble agar base (Difco) made up double strength (14gm /400 ml. of water). 10 ml. of a 20 % sterile solution of the required carbohydrate was added. For liquid medium, the agar was omitted. In ordinary minimal medium, glucose was the carbohydrate though other carbohydrates serving as carbon sources were also used.

Growth factor and streptomycin supplementation

Amino acids, pyrimidines, purines and vitamins were made up as stock solutions at 100 times the required concentration and autoclaved. When used in any minimal medium, amino acids, pyrimidines and purines were added to a final concentration of 20 mg/litre. Vitamins were added to a final concentration of 10 mg/litre. Streptomycin sulphate, when necessary, was added to minimal or nutrient agar medium at a final concentration of 600mg/litre.

Eosin methylene blue medium (EMB)

Dehydrated Bacto EMB Agar Base (Difco) was used. It contained :-

Bacto - peptone	10 gm/litre
Dipotassium phosphate	2 "
Bacto - agar	15 "
Bacto - eosin	0.4 "
Bacto - methylene blue	0.065 "

It was prepared as directed. The desired sugar was added to the medium at a final concentration of 0.4 %. Bacterial colonies which fermented a particular sugar stained a deep purple or green while non-fermenters appeared pink or white.

Endo agar medium

Dehydrated Bacto Endo Agar (Difco) was used. It was the Formula II of "Standard Methods" of the American Public Health Association. It contained:-

Bacto- peptone	10 gm/litre
Bacto- lactose	10 "
Dipotassium phosphate	3.5 "
Bacto- agar	15 "
Bacto- basic fuchsin	0.5 "
Sodium sulphite	2.5 "

It was prepared as directed. It was supplemented

with the required carbohydrate at a final concentration of 1 %. The base contained lactose but since the medium was used in studies with lactose non-fermenting Salmonella strains, its use for the other sugars was not impaired. Bacterial colonies which fermented a particular sugar stained blood red while non-fermenters appeared pink or white.

Semi-solid agar medium

It was composed of the following, per litre of water:-

Gelatin (Baltimore Biol. Lab.,Inc.(BBL), 1640 Gersuch Ave.,Baltimore 18, Maryland, U.S.A.)	- 80 gm.
Bacto peptone (Difco)	- 10 "
Beef extract (Difco)	- 3 "
Noble agar base (Difco)	- 4 "

The gelatin was dissolved in 600 ml. of water by heating to 50 - 60° C for 30 minutes. This was added to a 400 ml. solution of the remaining ingredients. The pH was adjusted to 7.2 - 7.4 and the medium sterilized by autoclaving (15lb. steam pressure for 15 mins.).

Liquid indicator medium for sugar fermentation

Two types of media were used. They differed in the indicators employed to detect acid production.

(i) Dehydrated Bacto Purple Broth Base (Difco). It contained:-

Proteose - peptone NO. 3	- 10 gm/litre
Bacto- Beef extract	- 1 "
Sodium chloride	- 5 "
Bacto- brom cresol purple	- 0.015 "

It was made up as directed.

(ii) Dehydrated Phenol Red Broth Base(BBL). It contained:-

Trypticase (Casein peptone)	- 10 gm/litre
Sodium chloride	- 5 "
Phenol red	- 0.018 "

It was made up as directed.

In both liquid media, the required carbohydrate was added as a sterile solution to a final concentration of 1%.

Sera

(a) Serum for immunological experiments

All serum was from pooled blood. The stumpy-tailed lizard, pig, horse, red kangaroo and human (blood group B) sera were kindly given by Mr. G.E.Schwab. In the case of rat, guinea pig, rabbit and mouse, blood was obtained by cardiac puncture and allowed to clot (30 mins. incubation at 37° C followed by 4-5 hours at 4° C). After the serum was freed from cells by centrifugation, it was aseptically removed and stored in small volume (2-3 ml .) at -20° C.

(b) Antiserum for the identification of Salmonella H antigens

Rabbit antiserum produced against the following Salmonella flagella antigens were used: phase 1 (i), phase 1 (b), phase 2 (1,2) and phase 2 (e,n,x). The anti-sera were generously provided by the Dept. of Bacteriology, Walter Reed Army Institute of Research, Washington, D.C.

3. Routine methods

Temperature of incubation 37° C except where otherwise stated.

Estimation of viable bacteria

The cultures were appropriately diluted in saline. 0.1 or 0.02 ml. aliquots of suitable dilutions were spread on duplicate nutrient agar plates. Colonies were enumerated after overnight incubation.

Replica plating

The procedure described by Lederberg and Lederberg (1952) using sterile velveteen pads and a metal block, was employed. It was used to transfer bacterial growth from a master plate to one or more other agar plates of similar or different composition.

Determination of sugar fermentative ability

Tubes containing 1 ml. amounts of liquid indicator medium were innoculated with bacteria from a loop which

had been brushed on a single colony. Fermenters or non-fermenters of a particular sugar were distinguished after overnight incubation. In the purple broth base medium, fermentation was indicated by a yellow colour, otherwise the medium remained purple. In the phenol red medium fermentation was indicated by a yellow colour, otherwise it was red.

Determination of flagella antigens of Salmonella

Bacterial cultures to be analysed for their antigens were initially made highly motile by two consecutive passages through semi-solid agar medium (in plates). The diagnostic procedure was identical to that described by Edwards and Ewing (1961) except that non-formalized broth cultures were used.

Test for production of colicine

To determine the synthesis of colicine either by single colonies or culture-streaks the bacteria were killed by chloroform vapour and then the plate was overlayed with about 10^8 bacteria of the indicator strain (W2586). The overlay consisted of soft agar seeded with bacteria. On overnight incubation colicinogenic bacteria were indicated by the presence of a clear zone surrounding the colonies, whereas non-colicinogenic ones did not have this zone.

Bacteriophage typing of S.typhimurium strains

A modification of the agar layer method for the assay

of phage was used (Adams, 1959). The test bacterial strain was incorporated as a layer (10^8 bacteria/plate) on a nutrient agar plate and allowed to harden. The various phage suspensions were spotted with a loop (6mm. diameter) onto the surface of the bacterial lawn. The plates were incubated 5-6 hours before reading the lysis patterns.

Purification of recombinants

This was accomplished by two single colony reisolations of the recombinants on the same medium as was used in their initial selection.

Characterization of biochemical markers in recombinants

Purified hybrids were spotted on nutrient agar (usually 25-30 hybrids/plate) and incubated overnight at room temperature. They were then replica plated onto differential media to determine the segregation of unselected markers. To determine the utilization of a particular carbohydrate, the recombinants were tested on minimal agar medium supplemented with the appropriate carbon source together with any nutritional requirements of the recombinants. Streptomycin resistance or sensitivity was assayed on nutrient agar containing streptomycin. In most instances, hybrids were further checked for their sugar markers in liquid indicator medium. This was always done with the hybrids to be tested for their mouse

virulence.

4. Experimental techniques

a. Selection of mutants and preparation of colicinogenic strains

Selection of nutritionally-exacting mutants

Two techniques were used. In one the mutagenic agent was ultra-violet (U-V) irradiation. In the other it was ethyl methane sulphonate (EMS). The initial strains of S.typhimurium were wild type, nutritionally non-exacting bacteria.

(1) U-V irradiation as mutagenic agent

The method was similar to that used by Adelberg and Myers (1953). An overnight broth culture of the wild type bacteria (0.02 ml.) was inoculated into 10 ml. of LM liquid medium and incubated for 2 hours with agitation. The logarithmic phase culture was diluted in the same medium (without glucose) to an optical density equivalent to 10^7 bacteria/ml. The suspension in a shallow layer, 1-2 mm. deep, was irradiated with U-V light from an Oliphant 25 watt germicidal lamp at a distance of 42 cm. for 1½ mins. Under these conditions 99.8 % of the bacteria were killed. Immediately 1 ml. of the irradiated suspension was incorporated in 6 ml. of LM agar and plated. Protective layers of 7 ml. and 5 ml. of similar agar above and below the suspension, were included. After

7 hours incubation, penicillin mixed with 6 ml. of LM agar was added. The concentration of the antibiotic was adjusted to give 200 units/ml. after diffusion into all the layers. The plates were refrigerated overnight before incubating them for 24 hours. LM medium (5 ml.) was added with sufficient penicillinase to give a final concentration of 500 units/ml. and the plates reincubated for 48 hours. All bacterial colonies appearing at this stage were marked and a final 5 ml. of nutrient agar was superimposed in a thin layer. Colonies appearing after 18 hours of incubation were carefully picked and analysed.

The nutritional deficiency of the mutants was determined by replica plating onto LM medium supplemented with various combinations of growth factors, as in Kaudewitz, Vielmetter and Fredrick-Freska (1958).

(2) EMS as mutagenic agent

The procedure was similar to that reported by Loveless and Howarth (1959). A nutrient agar plate was streaked to give a confluent sheet of growth upon incubation. The bacteria were scraped off into 10 ml. of 0.1M phosphate buffer (pH 7.0) and washed twice in the same diluent by centrifugation at 3,000 r.p.m./ 20 mins. The final pellet was resuspended in 10 ml. of 0.4M EMS and immediately kept at 37° C in a water bath for 20 mins.

Under these conditions, 99.7% of the bacteria were inactivated. An aliquot was withdrawn and diluted 1/100 into fresh broth. After overnight incubation, appropriate dilutions of the suspension were spread on nutrient agar plates. Nutritionally-exacting mutants were detected by replication to minimal medium. Their growth requirements were determined by replica plating on to minimal medium supplemented with various pools of growth factors as in "Selected methods in bacterial genetics" (1963). The composition of the pools were:-

Pool No.	1.	2.	3.	4.	5.
6.	adenine	guanine	cysteine	methionine	thiamine
7.	histidine	leucine	isoleucine	valine	lysine
8.	phenylalanine	tyrosine	tryptophane	threonine	proline
9.	glutamic	serine	alanine	aspartic	arginine

For example, if an isolate grew on pools 2 and 7, then its growth requirement was for leucine.

Selection of ara- mutants of S.typhimurium C5 His-

The culture was treated in an identical manner to that described previously for the selection of nutritionally-exacting mutants with EMS. However, the mutagen-treated suspension was manipulated differently after overnight incubation in broth. 10 ml. of this broth was centrifuged and washed twice in saline and the final pellet was resuspended in 5 ml. of minimal medium

supplemented with histidine and arabinose as the carbon source. The suspension was incubated for 5 hours and 0.1 ml. aliquots were added to tubes containing 3 ml. of the same medium with penicillin (300 units /ml.). After overnight incubation suitable dilutions of the penicillin-treated cells were then spread on EMB arabinose medium. Ara- mutants were picked and purified by two single colony reisolations on the same medium. The frequency of ara- by this procedure was 0.09 %. A single mutant was tested for its original markers to confirm its identity before using in experiments.

Selection of gal+ mutants of S.typhimurium Hfr SR305

40 ml. of overnight broth culture were centrifuged at 3000 r.p.m./20 mins. The pellet was resuspended in 0.5 ml. of saline. 0.1 ml. aliquots were then spread on a series of minimal agar plates containing galactose as the carbon source, supplemented with histidine. At the end of 3-4 days of incubation, galactose utilizing mutants were picked and purified by 3-4 single colony reisolations on the same medium. The frequency of gal+ mutants was about 2×10^{-10}. A single clone was checked for its original markers to confirm its identity before use.

Transfer of colicinogenic factors to non-colicinogenic bacteria

The S.typhimurium strains C5 His- and 37 were each

made colicinogenic for factor I by mixed growth with S.typhimurium cys D-36 (I). The transfer of factor E1 into C5 His- and 37 was also attempted by mixed growth with S.typhimurium cys D-36 (E1). The method was similar to that used by Ozeki et al., (1962). A loopful (from overnight broth cultures) of each of the colicinogenic and non-colicinogenic bacteria was inoculated into 5 ml. of fresh broth. After overnight incubation, the mixed culture was diluted and plated on selective medium (minimal supplemented with histidine for C5 His-; minimal supplemented with nicotinic acid for 37). Following 48 hours of incubation the plates were replica plated onto plates of the same medium. The colonies growing on the replicate plates were tested for colicine-producers and colonies corresponding to colicinogenic ones were picked from the master plate. These were purified by two single colony reisolations before testing for their original markers to confirm their identity.

C5 His- and 37 were each also made colicinogenic for factor E1 by mixed growth with E.coli CL 136(E1). Mixed growth was performed as described above. CL 136(E1) was killed by the technique described by Reynell (1962). That is, the culture was diluted 1/50 in a preparation of colicines B and K and incubated for 2 hours. Colicinogenic recipient clones were isolated by plating the

colicine-treated culture in a soft agar layer (Fredericq, 1957) and purified by single colony reisolations on minimal medium supplemented with the recipient's growth requirement as described for the isolation of factor I recipients. Their identity was confirmed by testing for their original markers.

Colicines K and B consisted of an equal mixture of the chloroformed broth cultures of cys C-7(K) and cys D-36(B), prepared as described by Fredericq (1957). Dissolved chloroform was removed by bubbling sterile air through the mixture.

b. Recombination Procedures

Standard mating procedure

Eighteen hour cultures of the bacteria to be mated were separately washed twice in saline and adjusted to give an approximate donor: recipient ratio of 1 to 5. The donor consisted of a broth culture while the recipient was a confluently streaked plate culture. Aliquots of the donor and recipient were directly plated together onto plates of selective medium. As controls, they were singly plated onto the same medium. The plates were incubated 48 - 72 hours before the recombinants were enumerated.

Colicinogeny-mediated recombination

The method of preparation of the mixture of colicin-

ogenic factor I and E1 donors was identical to that described by Ozeki and Howarth (1961). The recombination procedure per se was modified slightly depending on the frequency of recombination. When recombination was detectable the procedure reported by Ozeki and Howarth (1961) was used. When it was not detectable, then the donor and recipient mixture was incubated for 90 mins. Here the recipient cells in the mixture were 2-3 times in excess of donor cells instead of the 60 mins. incubation of an equal number of donor and recipient cells as was used by Ozeki and Howarth (1961). When recombination was not detectable with the Ozeki and Howarth technique it was also not detectable using the modified procedure.

Technique of transduction with phage PLT-22

The phage was propagated twice on S.typhimurium C5 wild type, in liquid medium, by the standard technique described by Adams (1959). The lysates were clarified by centrifugation. This was heated at 56° C for 30 mins. and tested for sterility by inoculating 0.1 ml. aliquots into broth and observing for turbidity after incubation. The phage was assayed on donor bacteria by the agar layer method (Adams, 1959). The titre was found to be about 7×10^9 plaque forming units/ml.

The transduction protocol was similar to that described by Furness and Rowley (1956). To 3 ml. of an

overnight broth culture of S.typhimurium C5 Leu-, 9 ml. of fresh broth was added and incubated for 1 hour. To 1 ml. of this culture an equal volume of phage suspension was added. The multiplicity of infection was about 10. As a control, broth was added instead of phage. The mixtures were incubated for 1/2 hour before diluting ten-fold with broth and reincubating for a further 3½ hours. They were then washed twice in LM medium (without glucose) and made up to 1/20 of the original volume. 0.02 ml. aliquots were spread in duplicate, on LM agar medium for the selection of prototrophs. The plates were incubated for 48 hours before the recombinants were counted.

c. Virulence titration of bacteria

Test animals

In the study of the genetic basis of virulence, male mice of the Bagg strain were used. The animals used in the experiments weighed between 16 - 22 gm.

In the study of the genetic and immunological comparison, between S.typhimurium C5 wild type and C5 Leu-, a strain of Swiss white mice was used. The animals used in experiments weighed between 18 - 22 gm. Male and female mice were used at random but each challenge group consisted of either males or females.

Virulence tests

Groups of 10 mice were challenged intraperitoneally

with either 0.2 or 0.5 ml. amounts of different dose levels of bacteria in saline. Immediately before injection viable counts were performed from suitable dilutions of the inoculum. The inoculum was prepared from 18 hour broth cultures. Cumulative deaths were recorded daily for 28 days and the 50 % lethal dose (LD50) was calculated by the method of Reed and Muench (1938).

Virulence screen test

In order to economize on the use of mice, a screening procedure for detecting the loss of virulence was used. Only a single dose level of about 5×10^3 bacteria per mouse was employed as the challenge in groups of 10 mice. Otherwise, the method was similar to the virulence tests.

d. Immunological and growth determining procedures.

Clearance of P^{32} - labelled bacteria by the reticulo-endothelial system of the mouse

The technique was essentially that described by Biozzi, Benacerraf and Halpern (1953). The bacteria were labelled as described by Jenkin (1962). Mice were injected intravenously with 2×10^8 labelled bacteria/mouse. The rate of clearance was expressed as the phagocytic index K.

$$K = \frac{\log C_1 - \log C_2}{t_2 - t_1}$$

where C_1 and C_2 are the concentration of the bacteria at

times t_1 and t_2 in mins. respectively.

Determination of the bactericidal power of fresh normal sera of different animals

Overnight cultures of bacteria grown in broth containing 25 % horse serum (inactivated at 56° C for 30 mins.) were diluted 1/5 into fresh medium of the same composition. They were incubated for 3 hours on a shaker. This was diluted to give about 5 x 10^4 bacteria/ml. and 0.1 ml. was added to 1 ml. of diluted serum from the various animals. All dilutions were made in 1/100 LH medium (without glucose). The bacterium-serum mixture was maintained at 37° C in a water-bath. At 0, 20, 40, 60 and 90 mins. aliquots were withdrawn and 0.02 ml. pipetted onto a quadrant of a nutrient agar plate using a standard dropping pipette. The plate was gently swirled and allowed to dry. The plates were incubated at 30° C for 18 hours before enumerating bacterial colonies.

Haemolytic complement assay

Complement was assayed according to the method of Kabat and Mayer (1961).

Intraperitoneal survival of bacteria

In all experiments 1,000-4,000 organisms were injected intraperitoneally into a series of mice. At time intervals mice were sacrificed and the peritoneum washed out

with fluid medium as described by Whitby and Rowley (1959). The viable count of the washings was then determined.

Opsonization of bacteria

An overnight broth culture of the organisms was diluted 1/10 in saline to give about 10^8 bacteria/ml. 0.5 ml. of this suspension was treated with an equal volume of diluted serum for 20 mins. at 4° C.

Serum absorption with bacteria

In some experiments sera used for opsonization had previously been absorbed with bacteria. For this purpose, 0.9 ml. of 3×10^9 bacteria/ml. of heat killed (two hours in a steam-bath) or viable bacteria were treated with an equal volume of undiluted serum for 30 mins. at 4° C. The serum was freed of bacteria by centrifuging at 5,000 r.p.m. for 20 mins.

Growth measurement of bacteria

An overnight broth culture of the bacteria was diluted 1/50 into fresh broth containing 10 % mouse serum (unheated) in Erlenmeyer flasks and gently agitated in a water bath at 37° C. At time intervals aliquots were withdrawn and their viable count determined.

Chapter 4

GENETIC AND IMMUNOLOGIC COMPARISON OF S.TYPHIMURIUM STRAINS C5 WILD TYPE AND C5 LEU-

As a preliminary to the study of the genetic basis of mouse virulence of S.typhimurium strains, the C5 wild type strain was treated with ultra-violet irradiation to obtain nutritionally-requiring mutants. Twenty such mutants were isolated and they were checked for their virulence. The mutants had different nutritional requirements and all of them, except one, had the same order of virulence as the wild type. This one mutant, C5 Leu-, was found to be between 6,000-10,000 times less virulent than the parental strain (Table 3). The Table also shows that the mean time to death of mice is much longer in the case of the mutant than in that of the wild type. Since the C5 Leu- strain was presumably obtained from the C5 wild type by mutation, it was thought of interest to determine in detail the reasons for the difference in mouse virulence between these two strains. However, it was first necessary to establish that the former was in fact derived from the latter. If it was otherwise, then a meaningful interpretation cannot be made for the difference in virulence between these two strains.

TABLE 3 VIRULENCE TESTS

Bacterial strain	LD50	Mean time to death[a]
S.typhimurium C5 wild type	5-10 bacteria	8 days
" C5 Leu-	6×10^4 "	17 "
Prototrophic transductants from the C5 Leu- strain (25 isolates tested)	$1-5 \times 10^4$ "	18 "

[a]Refers to mean time to death of mice at approximately the LD50 level.

In vitro characteristics and derivation of S.typhimurium C5 Leu-

On solid medium, the mutant was colonially indistinguishable from the C5 wild type. They both appear smooth by direct or transmitted light. When the resistance or sensitivity pattern of these two strains to a number of virulent and temperate bacteriophages of S.typhimurium was examined (Table 4), it proved to be identical. It was distinguishable from the pattern exhibited on S.typhimurium strains SR305 or M206 wild type. Even though a number of phages produced lysis on all the S.typhimurium strains, the lytic character on the C5 Leu- and the C5 wild type strains was identical and these were quite distinct from those produced on the M206 wild type and SR305 strains. In addition, C5 Leu- had an identical pattern of sugar fermentation to the C5 wild type (Table 5). They were distinguished from a number of other Salmonella strains. Slightly different results in the fermentation reactions of C5 Leu- and C5 wild type were reported in a publication (Krishnapillai et al., 1963). In it fermentation ability was determined by growth on EMB media containing various carbohydrates. The two sugars - arabinose and xylose were not fermented on EMB media by both C5 Leu- and C5 wild type, whereas in

TABLE 4 BACTERIOPHAGE LYSIS PATTERN ON S.TYPHIMURIUM STRAINS

Bacterial strain	B43h 49	TM7 Q1	SD7 Q1	PB. Jersey	TM A3	PB. BAUR	S162 Q1	D39h 41	H3 Q1	TM A4	TM A1a	PB. 3b	PB. 01	PB. 02	PB. 03
C5 wild type	-	+	-	-	+	-	-	-	+	-	+	+	+	+	+
C5 Leu-	-	+	-	-	+	-	-	-	+	-	+	+	+	+	+
M206 wild type	-	+	+	-	+	+	+	+	+	-	+	+	+	+	+
SR305	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+

+ = lysis produced; - = no lysis produced

TABLE 5 FERMENTATION OF CARBOHYDRATES BY S.TYPHIMURIUM STRAINS IN LIQUID MEDIUM

Bacterial strain	arabinose	xylose	rhamnose	maltose	inositol	mannitol	sorbitol	galactose	lactose	dulcitol
S.typhimurium SR305	+	+	+	+	+	+	+	-	-	-
" 37	+	+	+	+	-	+	+	+	-	-
" C5 wild type	+	+	-	+	-	+	+	+	-	-
" C5 Leu-	+	+	-	+	-	+	+	+	-	-
" M206 Ser-	+	+	+	+	-	n.d.	n.d.	+	-	n.d.
S.abony SW1444	+	+	+	+	+	+	+	+	-	n.d.

+ = fermented; - = not fermented; n.d. = not done

liquid media (Table 5) they were fermented. Why the C5 strains fail to give an indication of fermentation of the two sugars in EMB medium is not clear. That they do utilize arabinose and xylose as carbon sources in minimal medium was confirmed by their ready growth in such medium. At any rate, either on EMB media or in liquid media, the S.typhimurium strains C5 Leu- and C5 wild type have an identical reaction pattern. From these results it was concluded that the C5 Leu- strain was derived by mutation from the C5 wild type.

Transduction of prototrophy to S.typhimurium C5 Leu-

The next question of interest was whether the relative avirulence of the C5 Leu- strain was due to its inability to synthesize leucine. Bacon et al.,(1951) had earlier shown in their work with S.typhosa that purine, p-aminobenzoic acid and aspartic acid requiring strains, which were avirulent for the mouse, could be restored to full virulence by reversion to prototrophy or by incorporating the specific growth factor with the organisms during mouse challenge. Another method of restoring virulence to avirulent, nutritionally dependent bacteria was to transduce them to prototrophy with bacteriophage, as shown by Furness and Rowley (1956) with S.typhimurium adenine-dependent, avirulent strains. Hence the C5 Leu-

strain was transduced to nutritional independence with phage PLT-22. The transduction frequency for leu+ was found to be about 10^{-6} of treated recipient bacteria. 25 purified prototrophic transductant clones were then tested for their mouse virulence. Table 3 shows that the transduction of prototrophy to C5 Leu- fails to restore the full virulence of the C5 wild type. In addition, the mean time to death of mice challenged with the prototrophs is similar to the C5 Leu- strain. These results established that the leucine requirement of the mutant was unrelated to its avirulence. This means then that the relative avirulence of the C5 Leu- may be due to an independent mutation which occurred in the parental C5 wild type strain while selecting for the nutritionally requiring mutant. Therefore, the two strains were further studied to determine the existence of any growth-rate or antigenic differences.

Comparison of the in vitro growth characteristics of S.typhimurium C5 wild type and C5 Leu-

Before an animal dies of S.typhimurium infection it is known that the organism must have attained an enormous bacterial population. Avirulence could therefore be due to a slower growth rate of the mutant, as was in fact found by Hobson (1957) with str-r mutants of S.typhimurium which

were relatively avirulent. He found that the mean generation time was approximately 3-7 mins. longer for the str-r mutants than for the parental, virulent organisms. However, Fig. 1 shows that C5 wild type and C5 Leu- have very similar growth rates when grown in the presence of 10 % mouse serum.

Clearance of P^{32} - labelled bacteria by the reticulo-endothelial system of the mouse

The rest of the experimental work was designed to explore the possibility of any antigenic differences existing between the wild type and the mutant. It has been established that opsonic factors leading to the uptake of these bacteria by the reticulo-endothelial system are one of the major determinants of virulence and host susceptibility (Jenkin and Rowley, 1959). One would expect important antigenic differences of the parasite to be indicated by altered rates of phagocytosis. Therefore, the rate of clearance of S.typhimurium C5 wild type and C5 Leu- was measured in mice. The results are shown in Fig. 2, indicating that no significant differences were apparent. This implies then that the antigenic composition of the two strains is very similar.

Bactericidal power of fresh normal sera of various vertebrate species

Fresh normal serum has the potential capacity to

FIGURE 1 GROWTH CURVES OF S.TYPHIMURIUM C5 WILD TYPE AND C5 LEU-

The slopes of the regression lines between 1-5 hours were calculated before estimating the mean generation time (m.g.t.).

X___X C5 wild type. m.g.t. = 23.49 mins.

O___O C5 Leu-. m.g.t. = 23.41 mins.

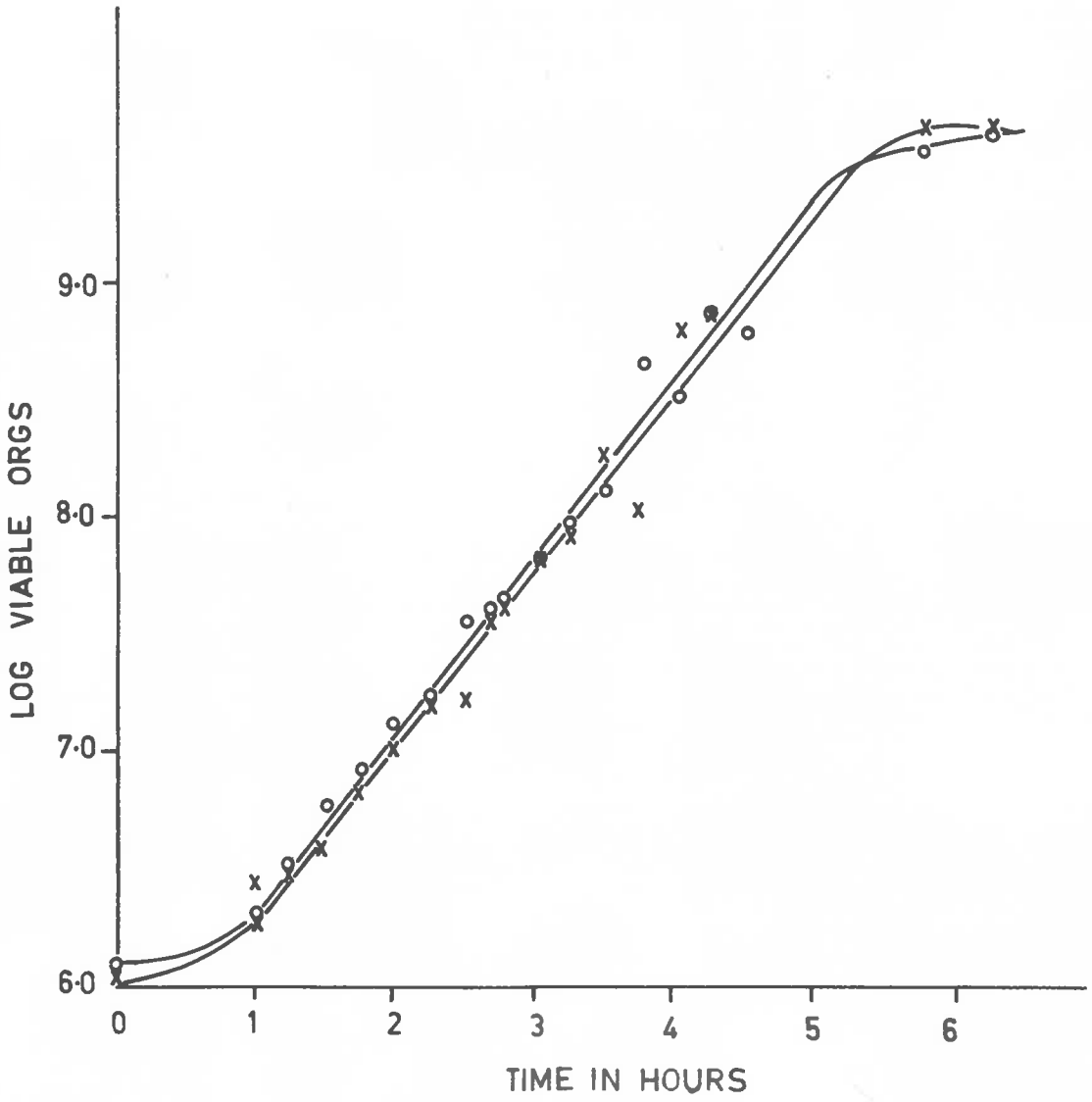
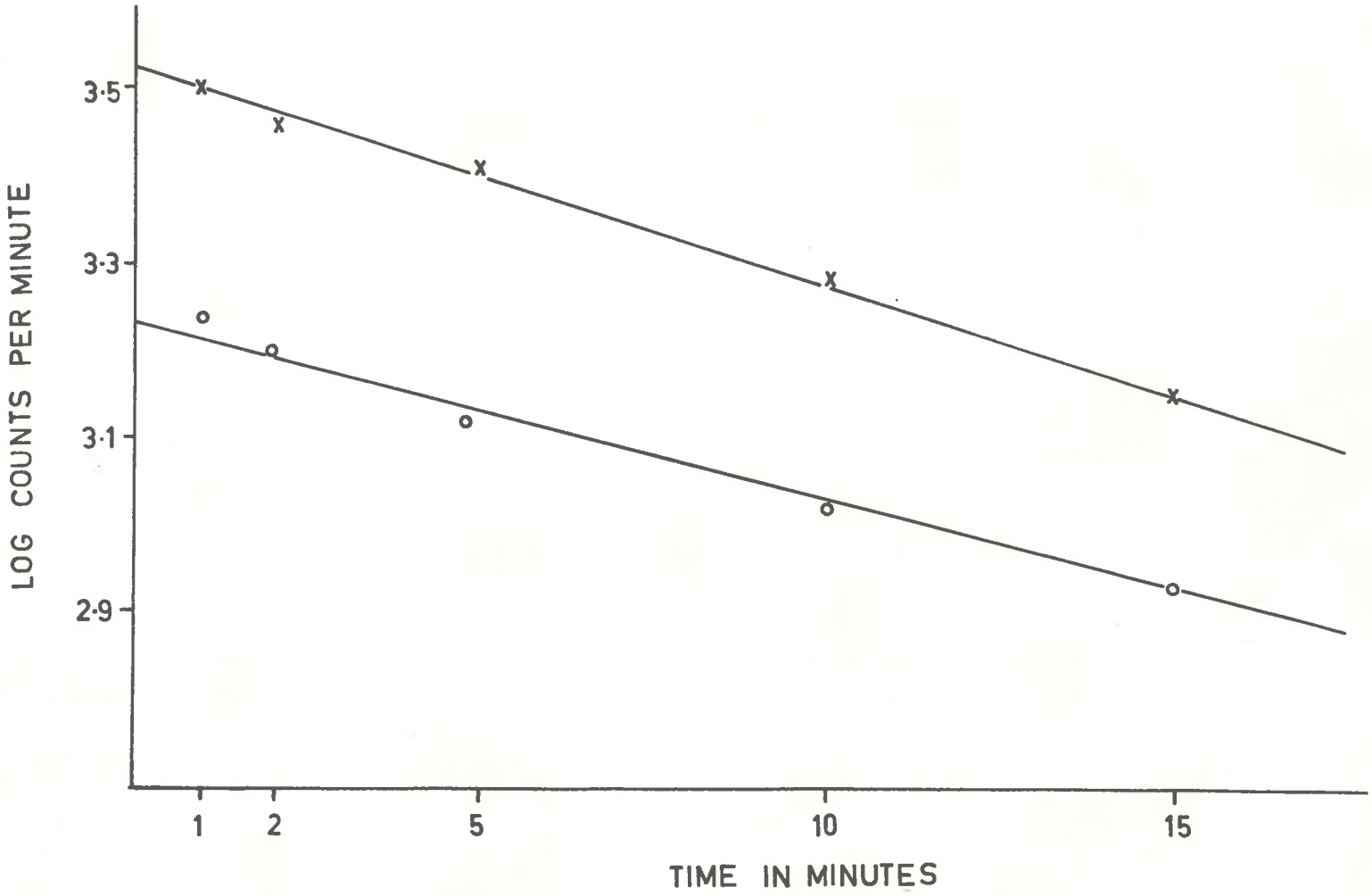

LOG VIABLE ORGS
9·0
8·0
7·0
6·0
0
1
2
3
4
5
6
TIME IN HOURS

FIGURE 2 INTRAVENOUS CLEARANCE OF P^{32} - LABELLED BACTERIA

The rate of phagocytosis is expressed as the phagocytic index K.

O___O C5 wild type. K = 0.020

X___X C5 Leu-. K = 0.026


LOG COUNTS PER MINUTE
3·5
3·3
3·1
2·9
1
2
5
10
15
TIME IN MINUTES

kill micro-organisms and the components partaking in this inactiviation are antibody and complement (Kabat and Mayer, 1961). The recent work of Michael, Whitby and Landy (1962) has confirmed the belief that in the presence of excess complement the efficiency of this system is proportional to the content of antibody directed against the "O" somatic antigen of the susceptible gram-negative bacteria. Hence the C5 wild type and C5 Leu- were tested against a variety of animal sera to determine whether a differential killing activity was exhibited by these sera. The data in Table 6 shows that no differences were obaerved even though complement was present in adequate amounts.

Intraperitoneal survival of S.typhimurium C5 wild type and C5 Leu- over a 28 day period

So far, no major difference between the C5 wild type and C5 Leu- had been established which may explain the lowered virulence of the C5 Leu- strain. Since the distinction in virulence between these strains was first established while titrating their mouse virulence, it was decided to follow the survival of the two strains after intraperitoneal injection into mice. Two groups of 120 mice each were injected intraperitoneally with about 3,000 bacteria per mouse of the wild type or of the mutant organism. At varying times, two mice were withdrawn from the pool, killed and peritoneal washouts done with 1.5 ml.

TABLE 6 SERUM BACTERICIDAL TESTS

Serum	Complement activity[a]	Bactericidal titre[b]	
		C5 wild type	C5 Leu-
Human (Blood group B)	120 units / ml.	½	¼
Stumpy-tailed lizard (*Trachysaurus rugosus*)	50 "	½	½
Rat	220 "	-	-
Guinea pig	690 "	-	-
Rabbit	70 "	-	-
Pig	60 "	-	-
Red kangaroo (*Macropus rufus*)	40 "	-	-

[a] Expressed as 50% Haemolytic units.

[b] Expressed as the dilution of serum producing 50% killing of the inoculum in 90 mins.

(-) Denotes no killing even at a ½ dilution of serum.

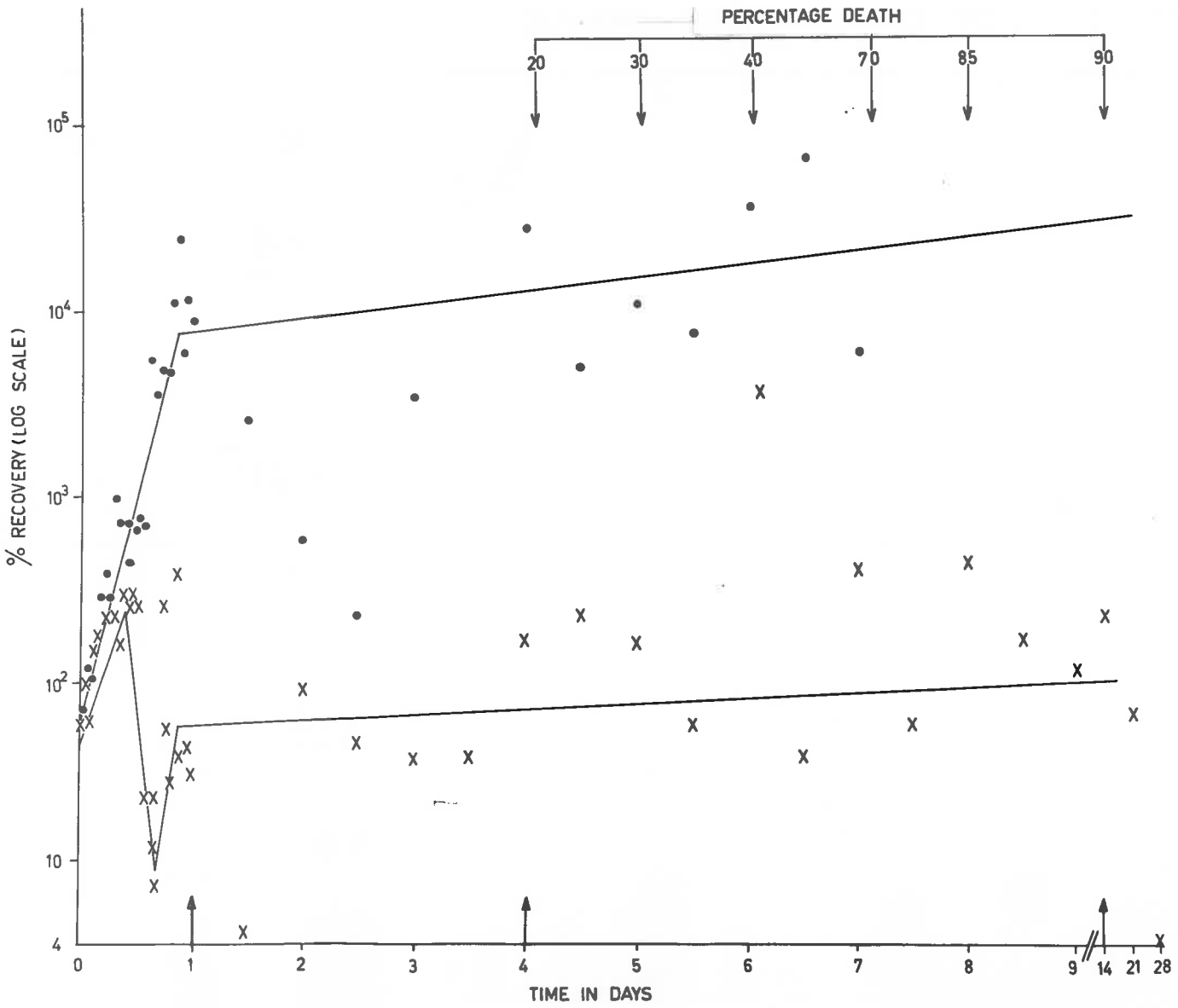
of Hanks' balanced salt solution containing 5 units/ml. of heparin (Weller et al., 1952). The total number of bacteria recovered was enumerated by viable count. The results of the two mice were averaged. Fig. 3 shows that the in vivo growth rates of the two strains are not distinguishable up to about 10 -12 hours after challenge. Between 12 and 24 hours the numbers of the avirulent strain decreased below the level of the challenge dose, but following this decrease remain at a fairly constant level for 21 days. In the next 7 days there is a gradual decrease. Mutant bacteria were still recoverable in very small numbers on the 28th day of infection. The numbers of mutant bacteria in the spleen and liver on the 14th day of infection was of the same order as found in the peritoneum at this time. On the other hand, the C5 wild type continued to increase rapidly for about 24 hours after challenge, at which time the difference in bacterial numbers between the two strains was about 100 fold. Though the wild type increased more slowly after the first day, it is to be noted that the actual number of viable bacteria only represents the survivors of the infection, since from about the third day mice began to die in increasing numbers and these have not been taken into account. The figures for percentage death refer to other experiments with the same challenge dose of the C5 wild type and are inserted

FIGURE 3 INTRAPERITONEAL SURVIVAL OF S.TYPHIMURIUM C5 WILD TYPE AND C5 LEU- OVER A 28 DAY PERIOD

The arrows on the time scale indicate the times at which sera were collected from mice, initially challenged with the mutant.

●___● C5 wild type.

X___X C5 Leu-.


PERCENTAGE DEATH
20
30
40
70
85
90
% RECOVERY (LOG SCALE)
10^5
10^4
10^3
10^2
10
4
0
1
2
3
4
5
6
7
8
9
14
21
28
TIME IN DAYS

to indicate the probable percentage deaths of mice which would have occurred in the present experiment if none had been withdrawn for washout.

Intraperitoneal survival of S.typhimurium C5 wild type and C5 Leu- pre-opsonized with sera from mice initially challenged with C5 Leu-

In this series of experiments a differential rate of killing of the two strains was sought for under conditions when they were pre-opsonized with specific antibody.

Unabsorbed 1-,4- and 14-day mouse serum

Six mice were withdrawn from the total pool of the experiment (shown in Fig. 3) 1, 4 and 14 days after the commencement of the experiment. The mice were bled and the sera from each group of six were pooled. Bacteria were opsonized with various dilutions of these sera and their survival in the peritoneum of normal mice over a period of two hours was studied. The serum taken from mice 1 day after infection with C5 Leu- behaved like normal mouse serum. That is, bacteria treated with such sera were not appreciably killed. This is similar to the survival of unopsonized bacteria. However, the 4-day serum greatly increased intraperitoneal killing to a degree similar to that obtained with 14-day serum. Figs. 4 and 5 indicate that no major difference in the intraperitoneal survival of the two strains exists when they

FIGURE 4 INTRAPERITONEAL SURVIVAL OF S.TYPHIMURIUM C5 WILD TYPE

O___O Unopsonized.

Δ——Δ Opsonized with normal mouse serum (final serum dilution of 1/10).

X___X Opsonized with 14-day serum (final serum dilution of 1/10). Half-life = 28 mins.

●___● Opsonized with 14-day serum (final serum dilution of 1/100). Half-life = 34 mins.

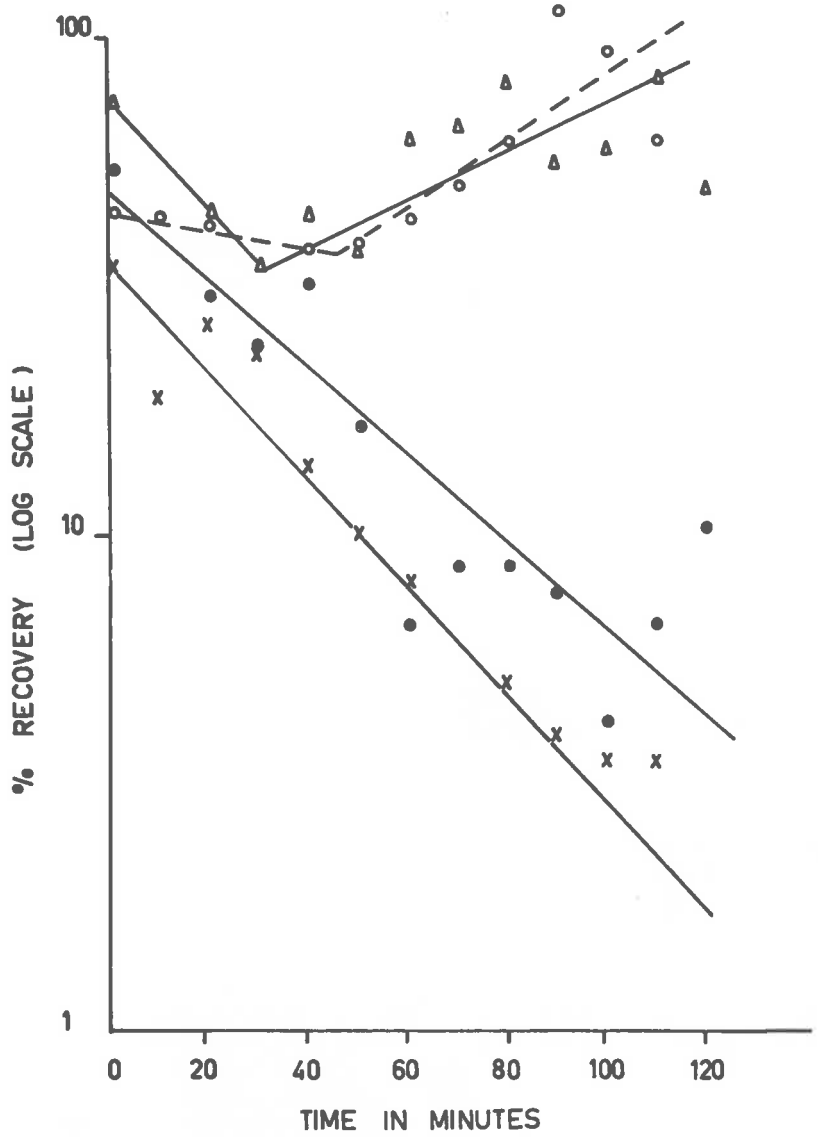

100
10
1
% RECOVERY (LOG SCALE)
0
20
40
60
80
100
120
TIME IN MINUTES

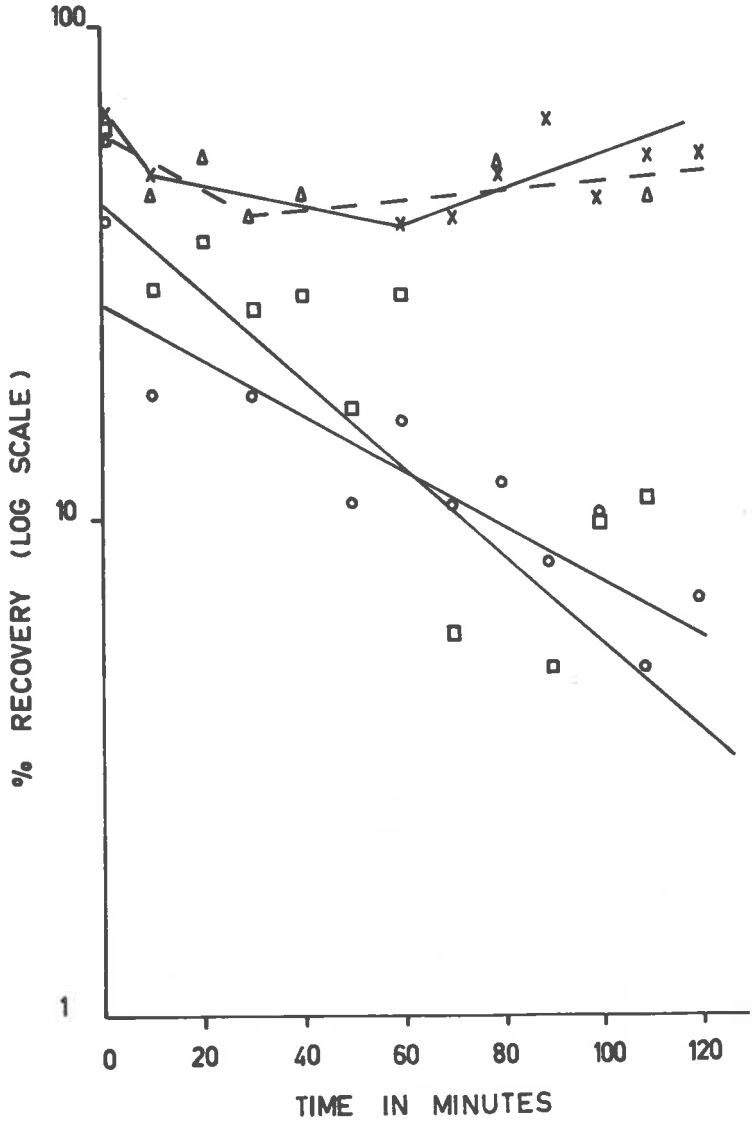
FIGURE 5 INTRAPERITONEAL SURVIVAL OF S.TYPHIMURIUM C5 LEU-

X—X Unopsonized.

Δ—Δ Opsonized with normal mouse serum (final serum dilution of 1/10).

□—□ Opsonized with 14-day serum (final serum dilution of 1/10). Half-life = 34 mins.

O—O Opsonized with 14-day serum (final serum dilution of 1/100). Half-life = 52 mins.


100
10
1
% RECOVERY (LOG SCALE)
0
20
40
60
80
100
120
TIME IN MINUTES

were pre-opsonized with unabsorbed serum. In Figs. 4, 5,6 and 7, the half-life refers to the time for 50 % reduction in bacterial recovery, starting from zero time. Therefore, the effect of pre-opsonization with absorbed sera was next studied.

14-day serum after absorption with C5 wild type bacteria

The pooled 14-day serum was absorbed with 0.5 ml. of 3×10^9 bacteria/ml. of viable wild type bacteria and this absorbed serum was used to preopsonize organisms before intraperitoneal injection into mice. It was found that a good deal of its activity against both strains had been removed (Fig.6). When, however, the same suspension of the C5 wild type was killed by heating at 100° C for two hours and a serum absorption then carried out under the same quantitative conditions as with the living bacteria, the results shown in Fig. 7 were obtained. That is, the half-life of the C5 wild type was 94 mins. whereas that of C5 Leu- was 24 mins. It was of interest that the 14-day serum had negligible amounts (less than 1/8) of agglutinating antibody against both living strains.

Discussion and Conclusion

The relative avirulence of S.typhimurium C5 Leu- was found to be unassociated with its growth-requirement for leucine. This was shown by the results of the transduction to prototrophy of C5 Leu-. It was not possible

FIGURE 6 INTRAPERITONEAL SURVIVAL OF S.TYPHIMURIUM C5 WILD TYPE AND C5 LEU- AFTER OPSONIZATION WITH 14-DAY SERUM PREABSORBED WITH VIABLE WILD TYPE BACTERIA

O___O C5 wild type. Final serum dilution of 1/20.
Half-life = 88 mins.

X___X C5 Leu-. Final serum dilution of 1/20.
Half-life = 94 mins.

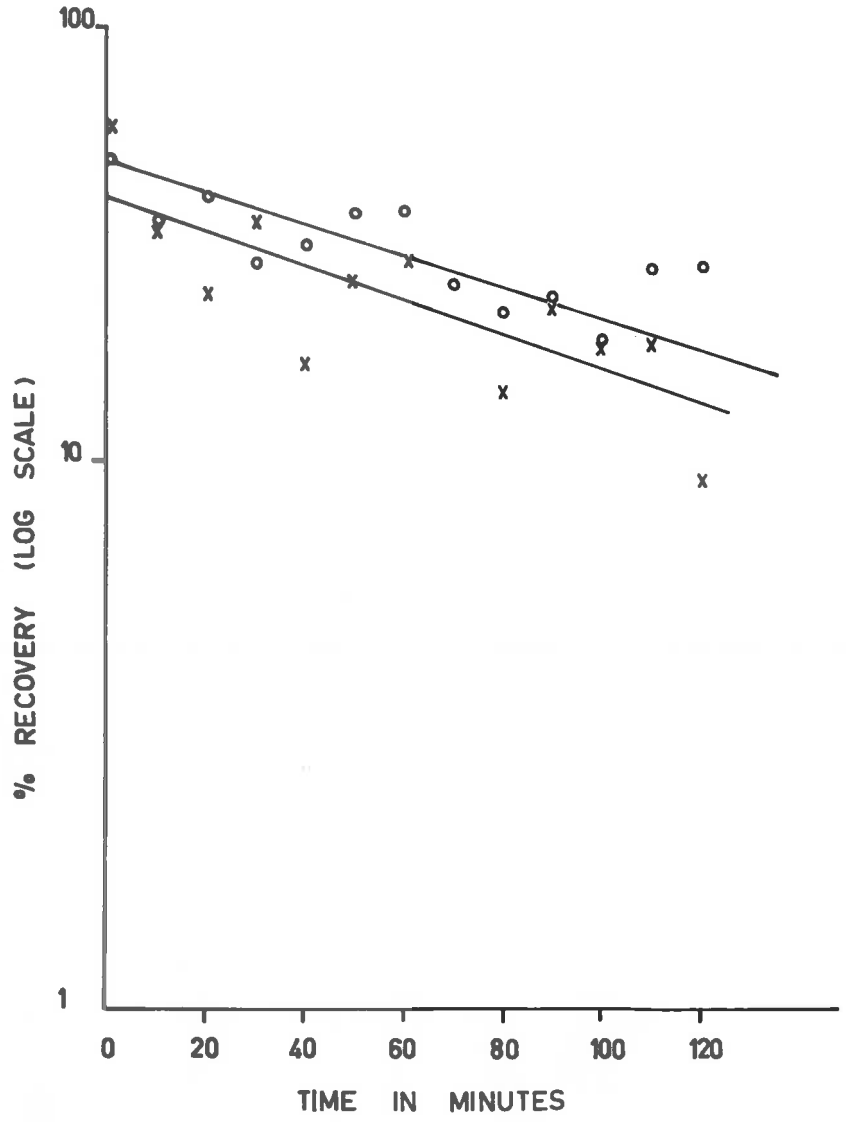
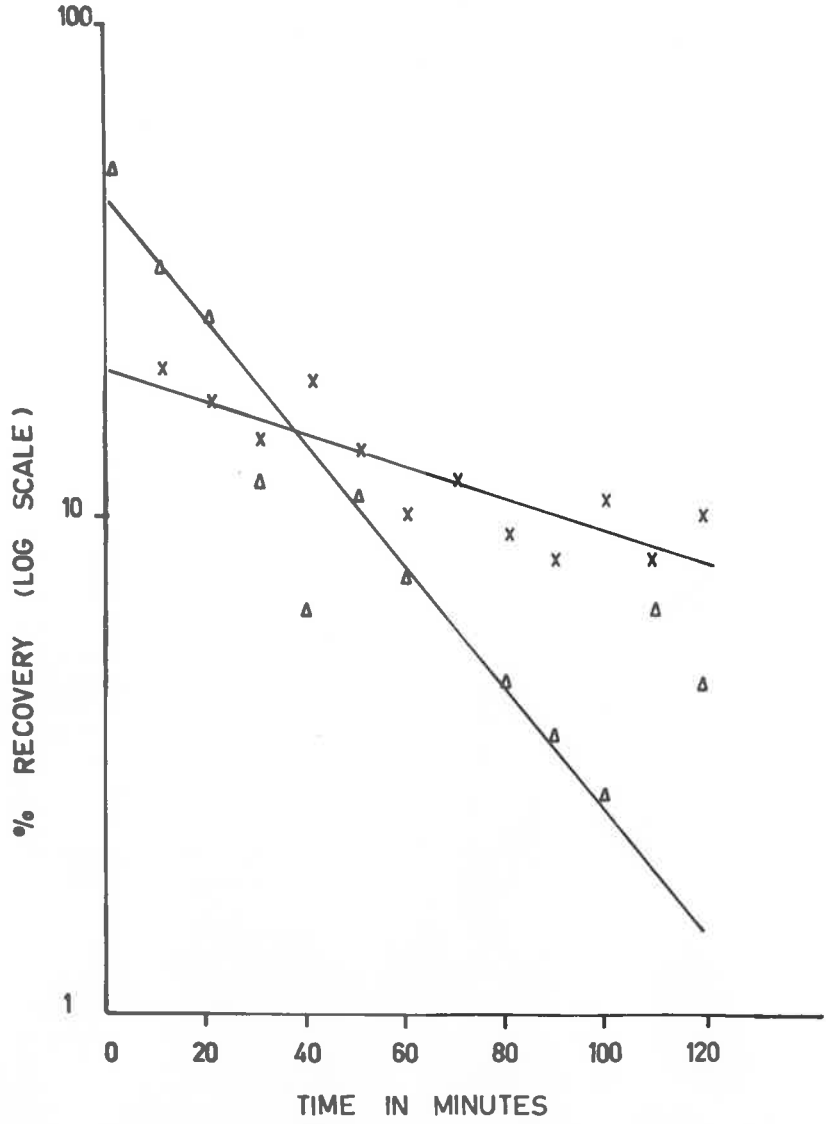

100
10
1
% RECOVERY (LOG SCALE)
0
20
40
60
80
100
120
TIME IN MINUTES

FIGURE 7 INTRAPERITONEAL SURVIVAL OF S.TYPHIMURIUM C5 WILD TYPE AND C5 LEU- AFTER OPSONIZATION WITH 14-DAY SERUM PREABSORBED WITH HEAT-KILLED WILD TYPE BACTERIA

X___X C5 wild type. Final serum dilution of 1/20.
Half-life = 94 mins.

Δ___Δ C5 Leu-. Final serum dilution of 1/20.
Half-life = 24 mins.


100
10
1
% RECOVERY (LOG SCALE)
0
20
40
60
80
100
120
TIME IN MINUTES

to detect readily any antigenic differences between the mutant and its virulent parent strain by studying the intravenous clearance of radioactive bacteria (Fig. 2) and bactericidal tests (Table 6). This contrasts with the findings of Jenkin (1962) who showed that the highly avirulent S.typhimurium strain M206 wild type was intravenously cleared ten times as rapidly as S.typhimurium C5 wild type. The failure to detect differences by the bactericidal tests may be explained if there were no natural antibodies to the antigen or antigens which differed in the C5 wild type and C5 Leu- strains in the variety of sera tested. The lack of detection in clearance rates may indicate a different reason for avirulence of the C5 Leu- strain in comparison to S.typhimurium M206.

Even though the in vitro growth rates of the two strains were found to be similar, their survival characteristics (Fig. 3) in the peritoneum indicated that the C5 wild type was able to increase in numbers rapidly, while the mutant remained at a fairly constant level. By about 24 hours, post-challenge , the numbers of wild type bacteria were 100 fold in excess of those of the mutant. It appears therefore, that the relative avirulence of the C5 Leu- strain may be due to its inability to increase rapidly in the mouse peritoneum which would be necessary for the eventual killing of the host.

Further differences between the two strains were found using immune sera. Serum from mice 4 days and 14 days after challenge with C5 Leu- was found to possess opsonic factors or antibodies as demonstrated by a greatly increased rate of phagocytosis and killing of bacteria pre-treated with dilutions of this serum. This activity was apparent against both the strains used but when the antibodies against the common heat-stable components of the "O" somatic antigens of these strains were removed by absorption with suspensions of heat-killed bacteria, the absorbed serum still possessed most of its opsonic activity towards the C5 Leu- strain. This suggests that there is a difference in some antigenic component other than the heat-stable "O" somatic antigen complex of the two strains.

The assumption was made that there was only a single change in the genetic structure in addition to the loss of the ability for leucine synthesis. If so, this change is responsible for the antigenic difference which consequently determines the difference in virulence between these two strains.

Chapter 5

ISOLATION OF CHROMOSOMAL DONORS OF VIRULENT STRAINS OF S.TYPHIMURIUM

When one studies the capacity of different strains of Salmonella to produce disease and kill mice, these bacterial strains could be conveniently subdivided into groups on the basis of their lethality for the host animals. Table 7 shows a representative number of strains and their lethal capacity for mice as measured by their LD50 values (the number of bacteria necessary to cause a 50 % killing in groups of challenged animals). The striking feature apparent from the Table is the small number of bacteria (less than 20 bacteria) of the S.typhimurium strains C5 wild type, Br1, 37 and 629 which can cause disease and kill mice. In contrast, to produce the same effect, 250,000 - 20,000,000 times more bacteria of the S.typhimurium strains M206 wild type, SW1292 and the S.abony strain SW1444 are required. The former group of strains is therefore, referred to as being highly virulent for the mouse. The latter are referred to as being avirulent. The S.typhimurium strains SR305 and C5 Leu- are partially virulent or avirulent since they occupy an intermediate position in the scale of LD50

TABLE 7 MOUSE VIRULENCE OF SALMONELLA STRAINS

Bacterial strain		LD50 (bacteria)
S.typhimurium	C5 wild type	5 - 10
"	Br1	<10
"	37	<20
"	628	<10
S.typhimurium	SR305	5×10^4
"	C5 Leu-	6×10^4
S.typhimurium	M206 wild type	5×10^6
"	SV1292	1×10^7
S.abony	SV1444	1×10^8

values shown in Table 7.

Preliminary studies

The mouse virulence of S.typhimurium strains may be studied by an examination of the genetics of virulence in crosses between virulent donors and avirulent recipients. It would involve an analysis of the recombinants arising from genetic crosses using genetically marked virulent donor and avirulent recipient combinations. Therefore, what was needed were chromosomal donors of virulent strains of S.typhimurium. A number of virulent strains were available for study (Table 7). Since a collection of different, singly-auxotrophic mutants of the C5 wild type strain were initially obtained (see Table 1), this strain was studied more closely.

Lack of genetic recombination within strain C5 or between strains C5 and M206 of S.typhimurium

In preliminary mating experiments it was found that prototrophic recombinants were not obtained (less than 10^{-9} of recipient bacteria) when complementarily-marked, single auxotrophs of C5 wild type were crossed with each other. Neither were prototrophic recombinants obtained in crosses between mutants of C5 wild type and complementarily-marked auxotrophs of the mouse avirulent strain, M206 wild type (Table 7). This indicated that strain C5 did not possess a sexual compatibility frame-work of

recombination similar to strain K-12 of E.coli. (Jacob and Wollman, 1961). Hence it was presumed that strain C5 was not F+ or Hfr in the sense of donor in E.coli K-12.

Attempts to isolate Hfr donor derivatives of S.typhimurium strain C5 in crosses with E.coli K-12 Hfr donors

As discussed in the literature survey in Chapter 2 Miyake (1962) was able to isolate an Hfr donor from an F- derivative of strain LT-7 (possessing the mutator gene) of S.typhimurium, by crossing it with the E.coli K-12 Hfr P4X-6. Similarly, an Hfr donor was isolated from strain TM-9Str-r-2 of S.typhimurium (Baron et al., 1963). Prior to obtaining the donors, it was shown by these workers that the S.typhimurium recipients were highly fertile with E.coli K-12 donors. Therefore, the C5 strain of S.typhimurium, either the wild type or its mutant derivatives, was examined to see whether it acted as a recipient (F-) for genetic material from E.coli K-12 donors. The rationale for this approach was the hope that eventually donor derivatives of strain C5 may be isolated, possessing the Hfr characteristic of E.coli K-12. Such donor strains of C5 could then be used in the genetic study of virulence.

Therefore, the E.coliK-12 Hfr W1895 (met-, lac+) was mated with the S.typhimurium C5 wild type (prototrophic, lac-), selection being made for lac+ recombinants on minimal medium containing lactose as the carbon source.

Though lac+ is transferred at very high frequency by this E.coli donor into E.coli K-12 F- strains (Baron et al., 1959a) no such recombinants were obtained (less than 10^{-10} of recipient bacteria). This indicated that C5 wild type did not readily recombine with the E.coli donor. Even though it failed to cross with W1895, a histidine requiring mutant of C5 wild type, C5 His- (his-, lac-) was mated with the E.coli K-12 Hfr P4X-6 (met-, lac+) for lac+ recombinants. The selective medium was minimal, supplemented with histidine plus lactose as the carbon source. Strain P4X-6 was the same donor that was used by Miyake (1962) and Baron et al., (1963) in their isolation of Hfr donors in S.typhimurium. They found that selection for lac+ resulted in the cotransfer of the Hfr characteristic into the Salmonella since the sex factor (F) was located close to lac+ and these markers were the last to be transferred by this Hfr. Again a negative result was obtained in the cross between P4X-6 and S.typhimurium C5 His-. This result was not surprising since even the transfer of the lead marker (lac+) from W1895 into strain C5 wild type was not observed. Similar results were obtained using a number of other E.coli K-12 Hfr strains and either other mutants of strain C5 wild type or strains Br1, 37 and 626 as recipients. This indicated that these virulent strains of S.typhimurium did not behave in the manner that was

displayed by the LT-7 F- mutator possessing or TM-9Str-r-2 strains of S.typhimurium.

F- infectibility of S.typhimurium strain C5

The next approach was to attempt to infect S.typhimurium C5 wild type with the E.coli K-12 sex factor (F), as was accomplished by Zinder (1960b) with S.typhimurium LT-2. He transferred the E.coli K-12 sex factor by culturing contiguously an E.coli F+ strain with strain LT-2 on EMB agar medium devoid of sugar. Red staining areas were observed at the junctions of growth of the E.coli and Salmonella. From these areas, he was able to isolate F+ bacteria of strain LT-2. From an initially F- infected LT-2 strain, Zinder subsequently isolated Hfr donors from this S.typhimurium. When this was attempted between S.typhimurium C5, either wild type or its mutant derivatives, with different E.coli F+ or Hfr donors, the red staining reaction was not observed. Neither was it seen when LT-2 wild type was tested with E.coli donors. Why the staining was not observed here is not known especially as Zinder noted its existence not only with strain LT-2 but also with 5 wild type strains of S.typhimurium. Since the basis of isolation of F+ derivatives of Salmonella was dependent on the observation of the staining reaction, it was not possible to isolate F+ derivatives in strain C5 of S.typhimurium.

Recipient capacity of S.typhimurium C5 strains with Salmonella Hfr donors

The experiments between S.typhimurium C5 and E.coli K-12 donors showed that this S.typhimurium strain was either unable to recombine with E.coli or that the methods that have been successfully used by others in different strains of S.typhimurium were inadequate in attempts to obtain chromosomal donors in C5. Not being able to obtain Hfr donors of virulent strains, it was wondered whether the genetics of virulence might be studied by an examination of hybrids arising from crosses between avirulent donors and virulent recipients.

Therefore, the strain C5 was examined to determine its mating potential with Salmonella donors. Two S.typhimurium Hfr donors were used. One was SR305 which was the Hfr isolated by Zinder from an F+ derivative of strain LT-2. The proximal markers injected by this Hfr were ile+ and leu+ (Zinder, 1961). The other was 15-11 which was isolated by Baron. The lead marker for this Hfr was pro+ (Baron et al., 1963). A number of single nutritional mutants of S.typhimurium C5 were crossed with these two Hfr strains. With the crosses involving SR305 a slightly modified mating procedure to that described in Materials and Methods was used. Overnight broth cultures of the donor and recipient were diluted 1/10 into fresh

broth and incubated for 2 hours. The rest of the mating procedure is as described in Materials and Methods. In crosses between SR305 and C5 recipients, the donor to recipient ratio was 1:1. With the other Hfr, the mating procedure described in Materials and Methods was used.

Table 8 shows that the C5 recipients were compatible with the donor strains tested. These experiments indicated that the mouse virulent S.typhimurium C5 strain was capable of acting as recipient for chromosomal material from S.typhimurium Hfr donors. The question was to use donor strains that were avirulent for the mouse. The LD50 value for the SR305 was 5×10^4 bacteria. This was not markedly less virulent than C5 recipients. (Tables 14, 15 and 16: Chapter 7). The Hfr 15-11 had an LD50 = $>10^6$ bacteria. This appeared as a suitable avirulent donor that could be used in crosses with S.typhimurium C5 recipients. Prior to such studies, recombinants from the cross :- Hfr 15-11 x C5 Met- were examined for the possession of unselected markers from the donor since extensive transfer of chromosomal material would be necessary in studies on the genetic basis of virulence. Therefore, 100 purified recombinants that had initially obtained the selected marker met+ were examined for the donor markers rha+ and inl+ . Not one had received rha+ or inl+. However, when a larger number of non-purified recombinants

TABLE 8 RECOMBINATION BETWEEN S.TYPHIMURIUM C5 RECIPIENTS AND S.TYPHIMURIUM HFR DONORS

Recipient strain	No. of prototrophic recombinants obtained with each Hfr donor	
	SR305	15-11
C5 Ile-	5,000	2,000
C5 Leu-	1,500	n.d.
C5 Try-	200	n.d.
C5 Ade-	0	n.d.
C5 Met-	n.d.	5,000
C5 His-	n.d.	100

The contraselecting marker against Hfr SR305 was his-. The contraselecting markers against Hfr 15-11 were pro-, arg-, ura-.and leu-. The number of recombinants obtained was approximate and the number of donor bacteria plated was between 5-10 x 10^8. In crosses with SR305 the recombinants were selected on unsupplemented LM medium. In crosses with 15-11 the recombinants were selected on unsupplemented minimal medium with glucose as the carbon source.

n.d. = not done

were studied, a few had received either rha+ or inl+. The low linked transfer was surprising especially for the markers met+ and rha+ since these are very close to each other (Falkow, 1963). This system for the study of the of the genetic factors controlling virulence was, therefore, abandoned.

Colicinogeny-mediated genetic recombination in S.typhimurium strains

This form of recombination has been performed by Ozeki and Howarth (1961) in strain LT-2 of S.typhimruium. In their system, the colicinogenic factors I and E1 were used as the promoters of chromosome transfer. An advantage of the system is that recombination for donor markers anywhere along the chromosome occurs with equal frequency. If colicinogenic donors of virulent strains of S.typhimurium were obtained then the genetics of virulence may be studied by colicinogeny-mediated recombination with non-colicinogenic, avirulent S.typhimurium strains. Therefore, the S.typhimurium strains C5 His- (LD50 = 2×10^3 bacteria; Table 14) and 37 (LD50 = <20 bacteria; Table 7) were each made colicinogenic for factors I and E1.

Transfer of colicinogeny into S.typhimurium strains C5 His- and 37

The transfer of colicinogenic factors I and E1 into these virulent strains of S.typhimurium is described in

Materials and Methods. Both the virulent strains were first determined to be non-colicinogenic as tested against the colicine indicator strain W2586. Table 9 shows that the results of the transfer of the I and E1 colicinogenic factors into strains C5 His- and 37. It indicates that the I and E1 factors can be successfully transferred into these strains. The transfer of E1 into C5 His- from the S.typhimurium cys D-36 (E1) donor was not done since the frequency of transfer of E1 by this donor into strain 37 was not detectable (< 0.05 %). The transfer of factor I into S.typhimurium 37 was very high and corresponds to that found by Ozeki et al., (1962) in the transfer of factor I either from S.sonnei P9 (I, E2) or S.typhimurium cys D-36 (I) into S.typhimurium cys D-36 Str-r by selecting the recipient in streptomycin medium. The transfer of factor I into S.typhimurium C5 His- occurred at a frequency of only 25 % which was about half the frequency of transfer into strain 37. This is equivalent to the frequency found by Meynell (1962) when the recipient was S.enteritidis and the donor was S.typhimurium ath-5 (I) which was another colicinogenic derivative of strain LT-2.

The transfer of factor E1 from S.typhimurium cys D36 (E1) into strain 37 was not detected although the E.coli K-12 colicinogenic donor CL136 (E1) transferred E1 into strain 37 at a frequency of 2 %. The greater

TABLE 9 TRANSFER OF COLICINOGENY FROM COLICINOGENIC DONOR STRAINS TO NON-COLICINOGENIC S.TYPHIMURIUM RECIPIENTS BY MIXED CULTURE

Donor of colicinogeny			Recipient of colicinogeny		
strain		colicine produced	strain	selected by	percentage colicinogenic[a]
S.typhimurium	cys D-36(I)	I	37	nutritional marker[b]	52
"	cys D-36(E1)	E1	37	"	<0.05
"	cys D-36(I)	I	C5 His-	"	25
"	cys D-36(E1)	E1	C5 His-	"	not done
E.coli	CL136 (E1)	E1	37	colicines B and K	2
"	CL136 (E1)	E1	C5 His-	"	0.2

For transfer of colicinogeny (see Materials and Methods).

[a] Refers to percentage of recipient colonies which were made colicinogenic.

[b] Refers to selection of recipient in minimal medium supplemented with its growth requirement (see Materials and Methods).

capacity of the E.coli donor to transfer E1 is analogous to that reported by Ozeki et al., (1962) where the same donor was 100 times more efficient in transferring the E1 factor into S.typhimurium than was transfer of E1 from colicinogenic to non-colicinogenic derivatives of the same S.typhimurium LT-2 strain. Perhaps as suggested by Ozeki et al., (1962) the presence of the sex factor (F) in the E.coli donor strain may assist in the greater transfer of the E1 colicinogenic factor.

It is also apparent from Table 9 that S.typhimurium C5 His- is a poorer recipient than S.typhimurium 37 in terms of its ability to receive either factor I or E1. It is not known why different strains of bacteria behave differently in their capacity to act as recipients for colicinogeny. It may be due to poor conjugation or instability of colicinogenic factors in certain recipient bacteria once they have been transferred.

Genetic recombination mediated by colicinogenic factors I and E1

At any rate, the possession of virulent, colicinogenic donors in S.typhimurium prompted an attempt at chromosomal recombination as has been done by Ozeki and Howarth (1961). The cross that was of particular interest in virulence studies was between S.typhimurium 37 as donor and S.typhimurium SW1292 as recipient. They differed in many genetic

markers (Table 1) which will obviously be useful in the study of the segregation of markers in any recombinants that may be selected. More importantly, strain SW1292 was avirulent (LD50 = 1 x 10^7 bacteria; Table 7) and hence well distinguishable from the virulence of strain 37 (LD50 = <20 bacteria, Table 7). Further crosses of interest were between S.typhimurium C5 His- as colicinogenic donor and S.typhimurium strains 175 (I) and M206 Ser- as recipients. The recipients in this case were avirulent (LD50 = 10^5 - 10^6 bacteria) although their precise LD50 levels were not initially determined. The C5 His- strain was virulent (LD50 = 2 x 10^3 bacteria; Table 14). The C5 Leu- recipient strain was only of intermediate virulence (LD50 = 6 x 10^4 bacteria; Table 7), and therefore not of great use in virulence studies using the C5 His- as the virulent donor since the distinction in virulence between these two strains was not very marked.

Prior to recombination experiments, the colicinogenic strains were checked for the stability of their colicinogenic characters by testing on the indicator strain (W2586) for colicine production. The method of preparation of the mixture of colicinogenic factor I and E1 donors was identical to that described by Ozeki and Howarth (1961). The technique of recombination is described in Materials and Methods. The results of recombination experiments are set

out in Table 10. It shows that genetic recombination was only detected between S.typhimurium C5 His- colicinogenic donors and S.typhimurium C5 Leu- as recipient. In every other combination, recombination for a number of different markers was not detectable. The frequency of recombination in the intrastrain C5 combination was comparable with the frequency obtained by Ozeki and Howarth (1961) in intrastrain recombination in S.typhimurium LT-2. The frequency was also similar to that found by Meynell (1962) in intrastrain colicinogeny-mediated recombination in S.enteritidis. The lack of interstrain recombination reported here is similar to that observed in interserotypic crosses involving S.enteritidis as donor and S.typhosa as recipient, although very low frequency (10^{-9}/recipient cell) recombination was obtained when the recipient was S.typhimurium LT-2 (Meynell, 1962). A probable reason for the failure of S.typhimurium 175 (I) to act as recipient may have been the fact that it was itself colicinogenic for factor I. The other recipient strains were all non-colicinogenic. Therefore, attempts were made to isolate a non-colicinogenic derivative from strain 175 (I). An overnight broth culture was diluted in normal saline to give about 10^4 bacteria/ml. The suspension was irradiated with ultra-violet light. Under these conditions there was about 10 % bacteria survival. It was then diluted into

TABLE 10 COLICINOGENY - MEDIATED GENETIC RECOMBINATION IN S.TYPHIMURIUM STRAINS

Donor mixture	Recipient	Selected markers	Selective medium	Frequency of recombination[a]
C5 His-(I) and C5 His-(E1)	C5 Leu-	leu+	minimal	3×10^{-7}
" "	M206 Ser-	ser+	"	$<2 \times 10^{-10}$
" "	175(I)	arg+	minimal+supplements[b]	$<2 \times 10^{-10}$
37(I) and 37(E1)	SW1292	ara+	minimal+supplements[c]	$<2 \times 10^{-10}$
" "	"	rha+	" "	$<2 \times 10^{-10}$
" "	"	mal+	" "	$<2 \times 10^{-10}$
" "	"	xyl+	" "	$<2 \times 10^{-10}$
" "	"	gal+	" "	$<2 \times 10^{-10}$
" "	"	pro+leu+	minimal	$<2 \times 10^{-10}$

The C5 His-(I) and C5 His-(E1) donor was contraselected by the his- or his- and str-s markers.
The 37(I) and 37(E1) donor was contraselected by the nic- and str-s markers.

[a] Expressed in terms of recipient bacteria.

[b] The medium was supplemented with isoleucine, proline, tryptophane, threonine and streptomycin with glucose as the carbon source.

[c] The medium was supplemented with proline, leucine and streptomycin with the selected carbohydrate as the carbon source.

fresh broth and incubated for 2 hours. Suitable aliquots were spread on nutrient agar plates to obtain 50 single colonies / plate on incubation. The ensuing clones were examined for non-colicinogenic bacteria by the test for production of colicine. With this method no non-colicinogenic derivatives (less than 0.1 %) of strain 175 (I) were obtained. It is, therefore, not known what effect factor I has on genetic recombination when the recipient is colicinogenic for this factor.

Discussion and conclusion

In this Chapter, some mouse virulent strains of S.typhimurium were studied with the view to obtaining chromosomal donors which could be used in the genetic determination of virulence. Attempts to produce Hfr donors were unsuccessful since the experiments with the E.coli K-12 Hfr donors indicated that these strains were unable to recombine with strain K-12.

It was found that the virulent strain C5 acted as recipient (F-) to genetic material from S.typhimurium Hfr donors. However, this system was not pursued further since one of the Hfr donors was not markedly less virulent than the C5 recipient strains, while the other Hfr donor rarely transferred more than the selected marker which meant that extensive regions of the bacterial chromosome could not be easily examined for associated changes in virulence.

Colicinogenic factors I and E1 were each successfully transferred into virulent S.typhimurium strains C5 His- and 37. Donor preparations of C5 His- were capable of chromosomal recombination in intrastrain combination. That is, with C5 Leu- as recipient. However, all interstrain combinations tested failed to produce recombination. The reasons for this inability were not determined. It would appear from the work of Ozeki and Howarth (1961) in S.typhimurium LT-2 and that of Meynell (1962) in S.enteritidis that intrastrain recombination mediated by colicinogenic factors can occur fairly readily, whereas interstrain or interserotypic recombination is either very low or absent (Meynell,1962). The reasons for the very low or absence of recombination in combinations other than intrastrain pairs is not known. At least the process of conjugation itself does not seem to be impaired since in experiments between colicinogenic S.enteritidis as donors and S.typhosa as recipient, Meynell (1962) reported that there was very high frequency transfer of the colicinogenic I and E1 factors in the absence of detectable recombination. The most likely explanation is that processes following conjugation are impaired.

In any case, the lack of colicinogeny-mediated recombination between virulent and avirulent strains of

S.typhimurium examined here, meant that this form of recombination could not be used for the genetic study of virulence.

Chapter 6

GENETIC RECOMBINATION BETWEEN S.ABONY HFR SW1444 AND S.TYPHIMURIUM C5 STRAINS

In the last Chapter it was seen that it was not possible to study the genetics of mouse virulence because a suitable recombination system between virulent and avirulent strains of *S.typhimurium* was not available. Table 7 (see last Chapter) shows that the LD50 of *S.abony* SW1444 is 1×10^8 bacteria and that of the *S.typhimurium* strains C5 wild type, 37 and 62G is less than 20 bacteria. It was therefore, decided to determine whether those virulent strains would act as recipients for genetic material from the *S.abony* Hfr donor SW1444. If they did, then it was hoped that the genetics of virulence might be studied using the avirulent donor and one of the virulent recipients. Hfr SW1444 is identical to *S.abony* Hfr SW1391 (Makela, 1963b). Strain SW1391 was one of 7 Hfr donor derivatives of *S.abony* which Mäkelä (1963a) isolated after *S.abony* strain 74 was made F+ by contact with an F+ donor of *E.coli* K-12. She found that SW1391 was very stable and that it behaved like a typical *E.coli* K-12 Hfr whereas the other Hfr donors were unstable. Dut to its stability, and the fact that it was the best in respect to the numbers of recombinants produced with *S.abony* recipients, SW1444 was

a technically favourable Hfr strain to examine, for its donor ability to virulent recipients of S.typhimurium.

Donor capacity of S.abony Hfr strain SW1444 with virulent strains of S.typhimurium.

In preliminary mating experiments it was found that SW1444 recombined with the S.typhimurium strains 37 and 62C to form nic+ recombinants at a frequency of about 10^{-5} per donor cell. The recombinants were selected on minimal, unsupplemented medium. The unselected marker inl of these recombinants was analysed by replica plating recombinants directly from the selective medium onto minimal medium with inositol as the carbon source. About 1 % of the recombinants were found to be inl+. When 05 His- (a derivative of S.typhimurium 05 wild type) was mated with SW1444, his+ recombinants were obtained at a frequency of about 10^{-6} per donor cell. The selective medium was unsupplemented, minimal medium. The unselected markers inl and rha of the prototrophic hybrids were analysed by replica plating recombinants directly from the selective medium with inositol or rhamnose as the carbon source. About 1 % of the hybrids were inl+ and about 50 % of the hybrids were rha+.

Encouraged by these preliminary results, which indicated that the S.abony Hfr SW1444 was capable of sexually recombining with the virulent strains of S.typhimurium, the 05 strain was chosen to study in greater detail its

recipient ability with SW1444.

Recipient capacity of S.typhimurium C5 strains with S.abony Hfr SW1444

A number of mutants of strain C5 (Table 1) were crossed with Hfr SW1444. The results are presented in Table 11. It shows that all the crosses were productive except when the recipients were C5 Cys- or C5 Arg-. The order of gene transfer by Hfr SW1444, as evidenced by the gradient of transmission, is compatible with that found by Mäkelä (1963a) with the same S.abony donor into S.abony recipients.

Transmission of the lead marker ara+ by SW1444 into C5 recipients

In recombination experiments with S.abony, Mäkelä (1963a) reported that the frequency of transmission of lead markers by different S.abony Hfr donors into S.abony recipients varied between 0.02 - 2 %. It was of interest therefore, to determine precisely what the frequency of transmission was of the lead marker ara+ from S.abony Hfr SW1444 into S.typhimurium C5. This was of interest since the frequency of intrastrain recombination is generally reported to be considerably higher than the frequency found in non-intrastrain recombination (Falkow et al.,1962; Baron et al., 1963). Therefore, the S.abony Hfr was mated with the S.typhimurium recipients C5 His- Ara- and the

TABLE 11 GRADIENT OF TRANSMISSION OF MARKERS FROM S.ABONY HFR SW1444 TO S.TYPHIMURIUM C5 RECIPIENTS

Recipient strain	Supplements added to minimal selective medium	Selected marker	Frequency per donor cell[a]
C5 His- Ara-	arabinose[b] and histidine	ara+	1×10^{-5}
C5 Leu-	glucose[b]	leu+	1×10^{-5}
C5 His-	glucose[b]	his+	2×10^{-6}
C5 Ile-	glucose[b]	ile+	4×10^{-7}
C5 Val-	glucose[b]	val+	2×10^{-7}
C5 His-	rhamnose[b] and histidine	rha+	2×10^{-8}
C5 His-	inositol[b] and histidine	inl+	2×10^{-9}
C5 Cys-	glucose[b]	cys+	$<2 \times 10^{-9}$
C5 Arg-	glucose[b]	arg+	$<2 \times 10^{-9}$

Crosses were performed by the mating procedure described in Materials and Methods. The Hfr contraselecting markers were arom- and met-. The frequencies refer to the approximate number of recombinants obtained by the standard mating procedure.

[a] Frequency of recombination

[b] Carbon source

hybrid C5 His+Ara-Str-r. The hybrid was obtained from the cross: S.abony Hfr x S.typhimurium C5 His- Ara-, initial selection being made for the his+ donor marker. The hybrid received the str-r marker as the unselected marker. The mating procedure was modified from that described in Materials and Methods so that mating took place in broth as was used by Mäkelä (1963a). The results obtained would then be comparable with hers. The procedure was as follows: 10 ml. of overnight broth culture of the donor and recipient were centrifuged and the pellet resuspended in fresh broth. The donor was adjusted to 2×10^8 bacteria/ml. and the recipient to 4×10^9 bacteria/ml. Equal volumes of the donor and the recipient were incubated together for 2 hours with agitation. Suitable aliquots directly from the mating mixture were then spread on selective media and recombinants were enumerated after 48 hours incubation. The frequencies of recombination were computed from the viable count of the donor and the recipient which was performed immediately prior to mating. The results are shown in Table 12. It is seen that the transmission of the lead marker ara+ occurs with a frequency of between 0.02 - 0.03 % into both the S.typhimurium recipients. In comparison, Makela (1963a) found that the same S.abony Hfr transferred the marker leu+ (which is closely linked to ara+) with a frequency of 2 % into S.abony

TABLE 12 FREQUENCY OF TRANSMISSION OF THE LEAD MARKER ARA+ BY S.ABONY HFR SW1444 INTO S.TYPHIMURIUM C5 RECIPIENTS

Recipient strain	Supplements added to minimal selective medium	Selected marker	Frequency per donor cell[a]
C5 His- Ara-	arabinose[b] and histidine	ara+	1.8×10^{-4}
C5 His+ Ara- Str-r[c]	arabinose[b]	ara+	3.0×10^{-4}

The mating procedure is described in the text. The donor to recipient ratio was 1:20. The Hfr contraselecting markers were arom- and met-.

[a] Frequency of recombination.

[b] Carbon source.

[c] Hybrid obtained from recombination of S.abony Hfr SW1444 with S.typhimurium C5 His- Ara-. It initially received the donor markers his+ and str-r.

recipients. Though this frequency is about 100 times higher than in the cross involving S.abony and S.typhimurium C5, the frequency obtained with the S.abony x S.typhimurium C5 crosses is still within the range of recombination frequencies observed in S.abony.

Linked transfer of markers from S.abony Hfr SW1444 into S.typhimurium C5 recipients

The next question was whether the hybrids, obtained from crosses between S.abony Hfr SW1444 and S.typhimurium recipients, had only received the selected marker or whether more extensive transfer had occurred. Recombinants arising from the three S.typhimurium recipients:- C5 His-, C5 His-Ara- and C5 Ile- were examined for the presence of unselected markers. Table 13 shows the segregation of unselected markers in the hybrids. It indicates that markers fairly widely separated may still be transferred into the C5 recipients, though the frequency of transfer of more than a single marker was low. The close linkage of the locus for rha+ to the his+ was unexpected but this may be a reflection of the uneven pairing compatibility along the chromosomes of the S.abony donors and S.typhimurium C5 recipients.

The Salmonella chromosome map is shown in Fig. 8, which indicates the relative position of the various markers used in the crosses: S.abony Hfr x S.typhimurium

TABLE 13 LINKAGE OF UNSELECTED MARKERS IN CROSSES USING S.ABONY HFR SW1444 AND S.TYPHIMURIUM C5 RECIPIENTS

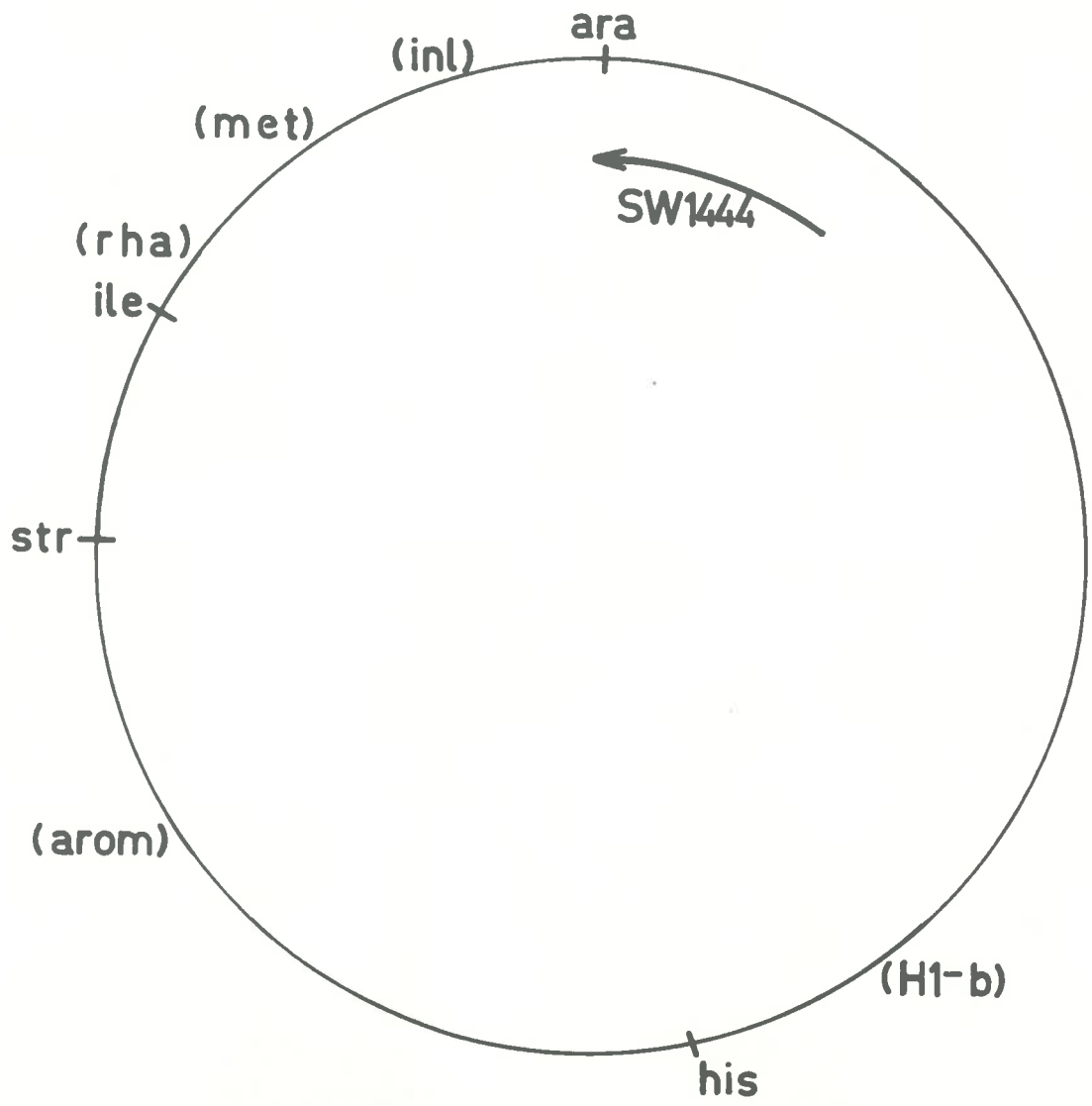
Recipient strain	Selected marker	Percentage frequency of unselected markers from donor					
		ara+	str-r	rha+	inl+	H1-b	H2-e,n,x
C5 His-	his+	n.d.	4	40	0	4	0
C5 His- Ara-	his+	4	2	24	0	n.d.	n.d.
C5 Ile-	ile+	n.d.	7	36	2	n.d.	n.d.

One hundred recombinants of the indicated selected marker were scored for the unselected markers shown. The flagella H antigen determinations were performed on 50 recombinants, obtained from the cross with the S.typhimurium C5 His- as recipient.

n.d. = not done

FIGURE 8 THE CHROMOSOME MAP OF SALMONELLA

It shows the location of the genetic markers used in recombination experiments between S.abony Hfr strain SW1444 as donor and S.typhimurium strains C5 His-, C5 His-Ara-, and C5 Ile- as recipients. The loci in parentheses indicate that the location of the marker in relation to the neighbouring markers has not been determined precisely. The inner line refers to the order of transfer of markers by the S.abony Hfr.


ara
(inl)
(met)
SW1444
(rha)
ile
str
(arom)
(H1-b)
his

recipients (C5 His-, C5 His-Ara- and C5 Ile-). Only the markers of relevance in the genetic study of virulence (see next Chapter) are shown. It has been constructed from the mapping done more extensively in Salmonella by Smith and Stocker (1962), Mäkelä (1963a), Falkow et al., (1962), and Falkow (1963). The relative positions of the various loci are based on the E.coli K-12 map (Jacob and Wollman, 1961). The relative positions of the markers are assumed for the purpose of identifying the regions of the chromosome which are associated with virulence in the strain C5 of S.typhimurium (see next Chapter).

Stability of hybrids obtained from crosses using S.abony Hfr SW1444 and S.typhimurium C5 recipients

The previous section showed that markers other than the selected marker may be detected in the hybrids. It was of obvious importance to determine whether the hybrids were stable and not segregating, partial heterozygotes. This phenomenon is common in interspecies hybrids studied in other enteric crosses (Baron et al.,1960 ; Falkow et al., 1962). It was particularly necessary to ascertain the stability of the hybrids since if they were segregating heterozygotes then the results of any hybrid's mouse virulence may be difficult to interpret. This is especially so since virulence titration experiments continue for 28 days. The mouse would obviously provide a selective

environment for the expression of the virulence gene complex of S.typhimurium C5.

Therefore, S.typhimurium C5 hybrids which showed a decrease in virulence (see next Chapter) were examined for the genetic stability of their other markers. Initially during the purification of the hybrids it was observed that they were colonially stable and did not appear to show any heterogeneity. Nor did they segregate colonial variants on non-selective medium (nutrient agar) as was the case with segregating heterozygotes (Baron et al., 1960; Falkow et al., 1962).

Before the stability of various donor markers in the hybrids were further tested, the hybrids were first grown in broth so that growth in such non-selective medium would encourage the segregation of donor and/or recipient alleles if the hybrids were unstable. Recombinants were either streaked or spread to obtain a large number of single colonies. Replica plating was used to transfer clones from nutrient agar onto differential media. The ile+ and His+ markers were studied by replica plating clones from nutrient agar onto minimal unsupplemented medium. The str-r marker was examined by replica plating from nutrient agar onto nutrient agar supplemented with streptomycin. With these markers the segregation of recipient alleles would be

indicated by lack of growth of certain clones on replicate plates when these plates are compared with the master plates. The rha+, inl+ or ara+ markers were examined by replica plating colonies from nutrient agar onto Endo rhamnose, Endo inositol or EMB arabinose medium, respectively. These carbohydrate markers were also tested by streaking hybrid cultures on the same indicator media. Due to the differential staining of colonies on such media it would be possible to detect any segregation. It was found that no marked instability was observed when 200 - 500 single colonies of each hybrid were examined(less than 0. 5% segregants).

In addition, in the case of the hybrids that were titrated for their mouse virulence, samples of the challenge inocula were also tested. Again there was no evidence of instability of the other markers.

Further confirmation that the recombinants were haploid was obtained from the following finding. This concerns the hybrids possessing the H1-b marker from the donor (Tables 14 and 17; Chapter 7). The hybrids before being tested for their flagella antigens were grown in broth and also cultured twice in semi-solid agar medium to enhance their motility. These media being non-selective would encourage the expression of segregant clones. That is, if the H1-b hybrids were heterozygous for the H1 locus

then they should segregate H1-b (from the donor) and H1-i (from the recipient) bacteria. Therefore, if such hybrids were tested in anti-b and anti-i sera then they should be agglutinated in both sera. But the 3 H1-b hybrids only agglutinated in anti-b sera and not in anti-i sera. However, because of the small number of hybrids receiving the H1-b and none receiving the H2-e,n,x marker from the donor, a larger number of hybrids were not available to study extensively the stability at the H1 and H2 loci.

Discussion and conclusion

In this Chapter, it was noted that the mouse avirulent S.abony Hfr strain SW1444 was capable of sexual recombination with the virulent S.typhimurium strains C5, 37 and 62G. Mutant derivatives of the C5 strain were studied in greater detail in recombination experiments with SW1444. A number of the C5 derivatives were found to be crossable with the S.abony donor. Recombination took place even for the distal marker (inl+) of the transfer sequence of the Hfr, although at very low frequency. In addition, markers fairly widely separated on the chromosome were still transferrable conjointly into strain C5. That C5 derivatives were in fact recipients to chromosomal material from the S.abony donor was suggested by the fact that the majority of hybrids still retained the marker characteristics of strain C5. By analogy with the E.coli K-12 fertility system, strain C5 is

therefore F- or female in comparison to SW1444. Analysis of the stability of donor alleles in S.typhimurium G5 recipients indicated that the hybrids were stable as evidenced by the lack of segregation of alleles. Therefore, they are true haploid recombinants. However, the reservation has to be made that the recombinants may be highly stable, persistent partial heterozygotes which were not detectable under the conditions of testing that were employed.

Chapter 7

THE VIRULENCE OF HYBRIDS FROM CROSSES BETWEEN S.ABONY HFR SW1444 AND S.TYPHIMURIUM C5 RECIPIENTS

In the last Chapter it was established that the S.abony Hfr strain SW1444 recombined with S.typhimurium C5 recipients. The fact that recombinants were obtained which had received extensive pieces of the donor chromosome meant that the opportunity now existed to study the genetics of mouse virulence of S.typhimurium C5. The LD50 of the S.abony was 1×10^8 bacteria (Table 14), whereas the LD50 of the S.typhimurium C5 recipients was 2×10^3 or less than 50 bacteria (Tables 14, 15 and 16). Therefore, the avirulence of the S.abony donor was distinguished from the virulence of the S.typhimurium C5 recipients by a factor of between 50,000-2,000,000 in terms of their LD50 values. The donor and the recipient were sufficiently alike antigenically to make meaningful comparisons of the virulence of the hybrids with the parental strains. That is, S.abony has the Kauffmann-White antigenic formula 4,5,12: b-e,n,x and S.typhimurium has 1,4,5,12: i-1,2. Therefore, the different classes of genetic hybrids shown in Table 13 were systemmatically examined for their mouse virulence. The virulence character was studied as an unselected marker in the hybrids.

TABLE 14 VIRULENCE OF HYBRIDS FROM THE CROSS: S.ABONY HFR SW1444 x S.TYPHIMURIUM C5 HIS-

Bacterial strain	Hybrid classes		No. tested	LD50 (bact)	Relative LD50[a]
	donor markers	recipient markers			
SW1444 (donor)	-	-	-	$1x10^8$	50,000
C5 His-(recipient)	-	-	-	$2x10^3$	1
hybrids	his[b]	str rha inl H1 H2	6	$<5x10^3$	1
	his[b] H1	str rha inl H2	2	$<5x10^3$	1
	his[b] str rha	inl H1 H2	2	$2x10^5$ $2x10^5$	100 100
	his[b] rha	str inl H1 H2	3	$<5x10^3$	1
	inl[b]	his str rha	1	$<5x10^3$	1
			2	$2x10^5$ $2x10^5$	100 100

The H1 and H2 markers of the inl+ hybrids were not tested.

[a]Expressed as the ratio of the LD50 of the strain under consideration to that of the LD 50 of strain C5 His-.

[b]Marker selected from donor.

TABLE 15 VIRULENCE OF HYBRIDS FROM THE CROSS: S.ABONY HFR SW1444 x S.TYPHIMURIUM C5 HIS-ARA-

Bacterial strain	Hybrid classes		No. tested	LD50 (bact)	Relative LD50[a]
	donor markers	recipient markers			
SW1444 (donor)	-	-	-	$1x10^8$	2,000,000
C5 His-Ara- (recipient)	-	-	-	$<5x10^1$	1
hybrids	his^b ara	str rha inl	3	$<5x10^3$	1
	his^b str	ara rha inl	1	$1x10^5$	2,000

[a] Expressed as the ratio of the LD50 of the strain under consideration to that of the LD50 of strain C5 His- Ara-.

[b] Marker selected from donor.

TABLE 16 VIRULENCE OF HYBRIDS FROM THE CROSS: S.ABONY HFR SW1444 x S.TYPHIMURIUM C5 ILE-

Bacterial strain	Hybrid classes: donor markers	Hybrid classes: recipient markers	No. tested	LD50 (bact)	Relative LD50[a]
SW1444 (donor)	-	-	-	1×10^8	2,000,000
C5 Ile-(recipient)	-	-	-	$< 5 \times 10^1$	1
hybrids	ile[b]	str rha inl	19	$< 5 \times 10^3$	1
			1	1×10^5	2,000
	ile[b] rha	str inl	16	$< 5 \times 10^3$	1
	ile[b] str	rha inl	2	$< 5 \times 10^3$	1
				2×10^7	400,000
			4	1×10^6	20,000
				3×10^5	6,000
				7×10^5	14,000
	ile[b] str rha	inl	1	6×10^5	12,000
	ile[b] inl	str rha	1	$< 5 \times 10^3$	1
	ile[b] rha inl	str	1	1×10^5	2,000

[a] Expressed as the ratio of the LD 50 of the strain under consideration to that of the LD50 of strain C5 Ile-.

[b] Marker selected from donor.

Observations on the determination of virulence

The donor strain of S.abony is completely avirulent for the mouse (Table 14), 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 2-3 days (endotoxic death), no further deaths occurring after this period. As little as a five-fold 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 as is shown in Tables 14,15 and 16. The mortality in groups of mice injected with even small doses of bacteria of the C5 strains is progressive with time, commencing 4-5 days after challenge and continuing thereon until the termination period of the experiment, usually 28 days. A characteristic difficulty in LD50 determinations with the C5 strains is that of obtaining satisfactory 50 % end-points. In the case of S. typhimurium C5 His-Ara-, for example, a challenge dose of 60 organisms produced 7/10 deaths, 600 organisms resulted in 6/10 deaths and 6,000 organisms also yielded 6/10 deaths. While these results can be taken to indicate that the LD50 of the C5 strains is less than 50 organisms, occasionally a dose-response mortality curve is obtained from which the

LD50 can be calculated as in the case of the C5 His- strain listed in Table 14. All the hybrids of partial avirulence which were studied, however, display a mortality curve that is dose dependent.

Virulence screening

Instead of determining the LD50 of each of the many hybrids between S.abony Hfr SW1444 and S.typhimurium C5 recipients, a screening procedure for the initial detection of loss of virulence was used (see Materials and Methods). Using 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 - 100 % mortality occurred in these mice; hence these hybrids were considered to be virulent. In Tables 14, 15 and 16, the LD50 of such hybrids is entered as being $< 5 \times 10^3$ bacteria. On the other hand, the S.abony donor or any partially avirulent hybrid produced 0-10 % mortality with the same dose level of challenge organisms. Only those hybrids exhibiting a partial avirulence by the screening procedure were tested to determine their precise LD50 values (Tables 14,15,16 and 17). The last column in these Tables expresses as a ratio the LD50 of the various hybrids to that of the LD50 of the virulent recipient strain of S.typhimurium. This is used as the measure of the decrease in virulence of hybrids.

For simplicity, the relative LD50 of the hybrids found to be virulent by the virulence screening procedure, is indicated as equal to 1 in the last column of Tables 14,15 and 16. The LD50 value of the S.abony Hfr strain is included in each Table for comparative purposes.

The virulence of hybrids

(a) Hybrids from the cross:- S.abony SW1444 x S.typhimurium C5 His-

The first set of hybrids that were tested for their virulence were the ones from the S.typhimurium C5 His- recipient (Table 14). The Table sets out the number of each kind of hybrid that was tested. It also shows the donor markers that were detected in the hybrids. The results of the virulence tests show that hybrids receiving only the his+, his+H1-b, or his+rha+ markers from S.abony are still virulent. But both the hybrids receiving the his+str-r rha+ region from the donor are partially avirulent. The transfer of the in1+ marker alone produced two hybrids that were partially avirulent. However, one hybrid receiving the in1+ marker was still virulent. All the partially avirulent hybrids have a LD50 = 2×10^5 bacteria which in comparison with the parental S.typhimurium recipient is 100 times less virulent.

(b) Hybrids from the cross:- S.abony Hfr SW1444 x S.typhimurium C5 His-Ara-

Table 15 shows the analysis of the virulence of hybrids from the recipient S.typhimurium C5 His-Ara-. The transfer of the donor segment his+ ara+ into hybrids can be seen to have no effect on their virulence. The one hybrid which received the his+ str-r segment is partially avirulent (LD50 = 1 x 10^5 bacteria). Its LD50 is about 2,000 times less than that of the parental C5 His-Ara- strain.

The results shown in Tables 14 and 15 suggest that the region involved in the determination of the difference in virulence between the S.abony donor and the C5 recipients lies within the chromosomal segment his---str---inl (see Fig. 6; Chapter 6).

(c) Hybrids from the cross:- S.abony Hfr SW1444 x S.typhimurium C5 Ile-

Since the donor genetic material, whose transfer into the S.typhimurium C5 recipients reduces its virulence, lies in the chromosomal segment his---str---inl, it was decided that this segment should be looked into more closely. Therefore, the C5 Ile- strain was used as recipient in matings with the S.abony donor. The various classes of hybrids isolated from this cross (which are shown in Table 13) were then tested for their mouse virulence. The results are shown in Table 16. Of the 20 hybrids that had

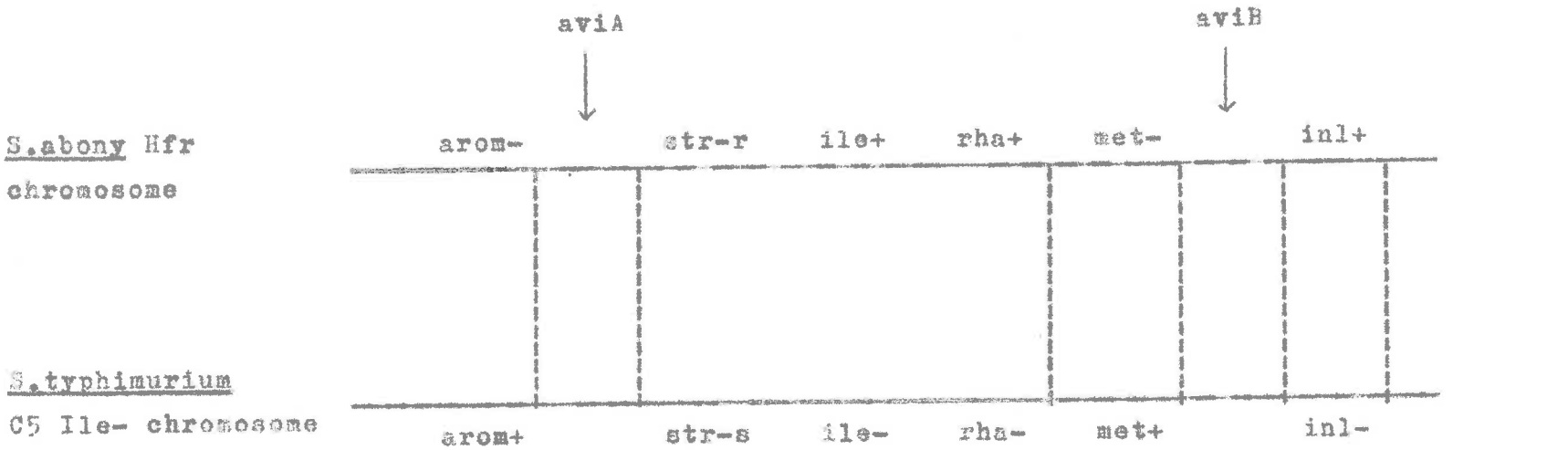
received only the selected marker (ile+), 19 were still virulent. One of these hybrids was, however, partially avirulent (LD50 = 1 x 10^5 bacteria). All sixteen hybrids possessing the closely linked donor markers ile+ rha+ were also still virulent. The one hybrid possessing the donor markers ile+ inl+ was still virulent. Hence the transfer of ile+ alone or ile+rha+ or ile+inl+ by the S.abony donor fails to produce any change in virulence, thereby eliminating these individual segments from being associated with the observed virulence alteration. However, 4 of the 6 hybrids inheriting the donor markers ile+str-r are partially avirulent. Their LD50 values range from 3 x 10^5 - 2 x 10^7 bacteria. The remaining 2 hybrids of this genetic class are virulent. The hybrid receiving the donor segment ile---str-r---rha displays partial avirulence (LD50 = 6x10^5 bacteria). Similarly, the hybrid inheriting the donor markers ile+ rha+ inl+ is partially avirulent (LD50 = 1x10^5 bacteria). These results together with those shown in Tables 14 and 15, indicate an association of the loci for str and inl with alterations in virulence. It is to be noted that str and inl are unlinked (Fig. 8; Chapter 6).

Genetic interpretation of the segregation of partial avirulence in the hybrids

Although the acquisition of partial avirulence by the hybrids is always associated with either the str or the inl

marker, 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 inl marker. It becomes necessary, therefore, to look at the process of selection of recombinants in order to explain this segregation. For convenience, the cross: S.abony Hfr SW1444 x S.typhimurium C5 Ile- is illustrated in Fig. 9. It will be seen later that the situation is applicable to the segregation of partial avirulence amongst the hybrids arising from the other two S.typhimurium C5 recipients as well. Fig. 9 diagrammatically illusrates the pairing of the chromosomes of the S.abony donor and the S.typhimurium C5 Ile- recipient prior to recombination. The donor is arom- met- while the 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 inl for the formation of inl+ recombinants. Now, if it is assumed that an avirulence (aviA) locus is located on the chromosome as shown, then some among those hybrids containing the donor chromosomal region ile+ str-r would be avirulent while others would be virulent (Table 16) depending on whether the crossover took place to the right or left of this locus. Similarly, some hybrids inheriting the donor marker inl+ would be avirulent while others would be

FIGURE 9 REPRESENTATION OF THE CHROMOSOMES OF S.ABONY HFR SW1444 AND S.TYPHIMURIUM C5 ILE-



The crossovers required at the indicated locations, for the formation of prototrophic or inl+ recombinants by genetic recombination, which could lead to the segregation of the avirulence loci.

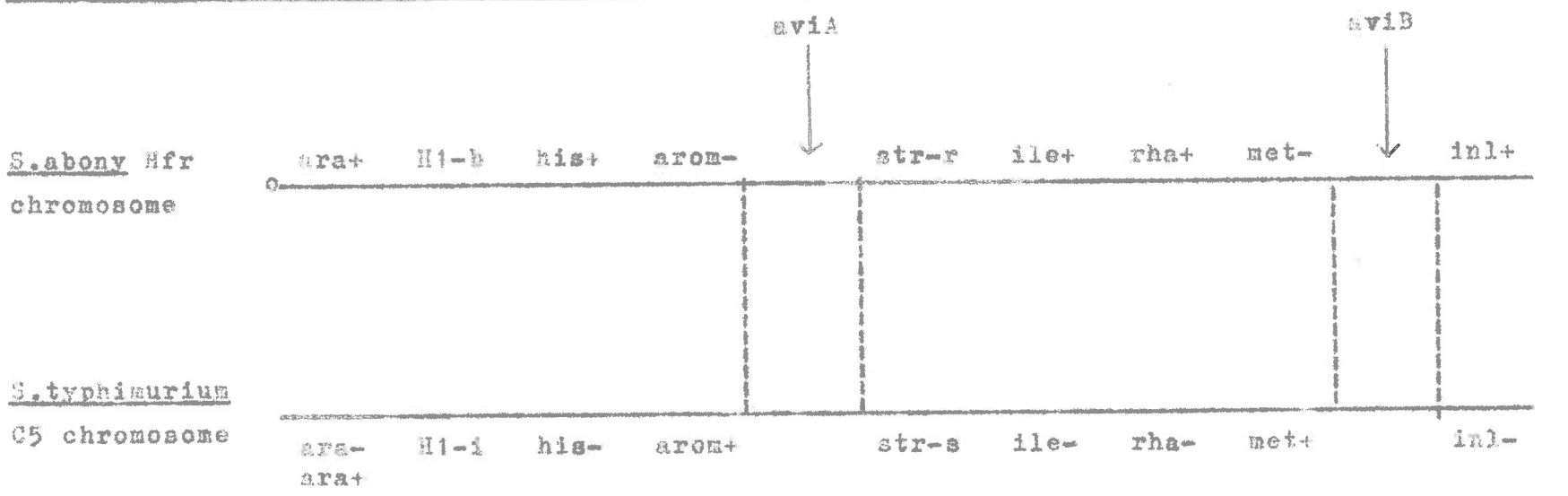
----- = crossovers

virulent (Table 16) depending on the occurrence of a cross-over to the right or left of the position of aviB as shown in Fig.9. The crossover between rha and met is not associated with virulence since all the hybrids inheriting the donor markers ile+ rha+ are still virulent. It can now be seen, as shown in Fig.10, that the depiction of two loci controlling avirulence in the S.abony strain which are transferrable to S.typhimurium, is compatible with the experimental findings. In the light of this postulation, it is concluded that the hybrids receiving the donor markers his+ str-r rha+ arising from the cross using S.typhimurium C5 His- as recipient (Table 14), are partially avirulent because they had inherited the S.abony allele at the aviA locus. Similarly, the 2 hybrids receiving the donor marker inl+ are partially avirulent because they had inherited the S.abony allele at the aviB locus. The hybrid receiving the donor markers his+ str-r from the cross using S.typhimurium C5 His- Ara- as recipient (Table 15) is partially avirulent since it inherited the donor allele at the aviA locus. The location of aviA and aviB on the Salmonella chromosome that was constructed in the previous Chapter, is shown in Fig.11.

Backcrossing a partially avirulent hybrid with the same S.abony donor

It is obvious from an examination of Tables 14,15 and

FIGURE 10 REPRESENTATION OF THE CHROMOSOME OF S.ABONY HFR SW1444 AND THE COMPOSITE CHROMOSOME OF S.TYPHIMURIUM C5 RECIPIENTS

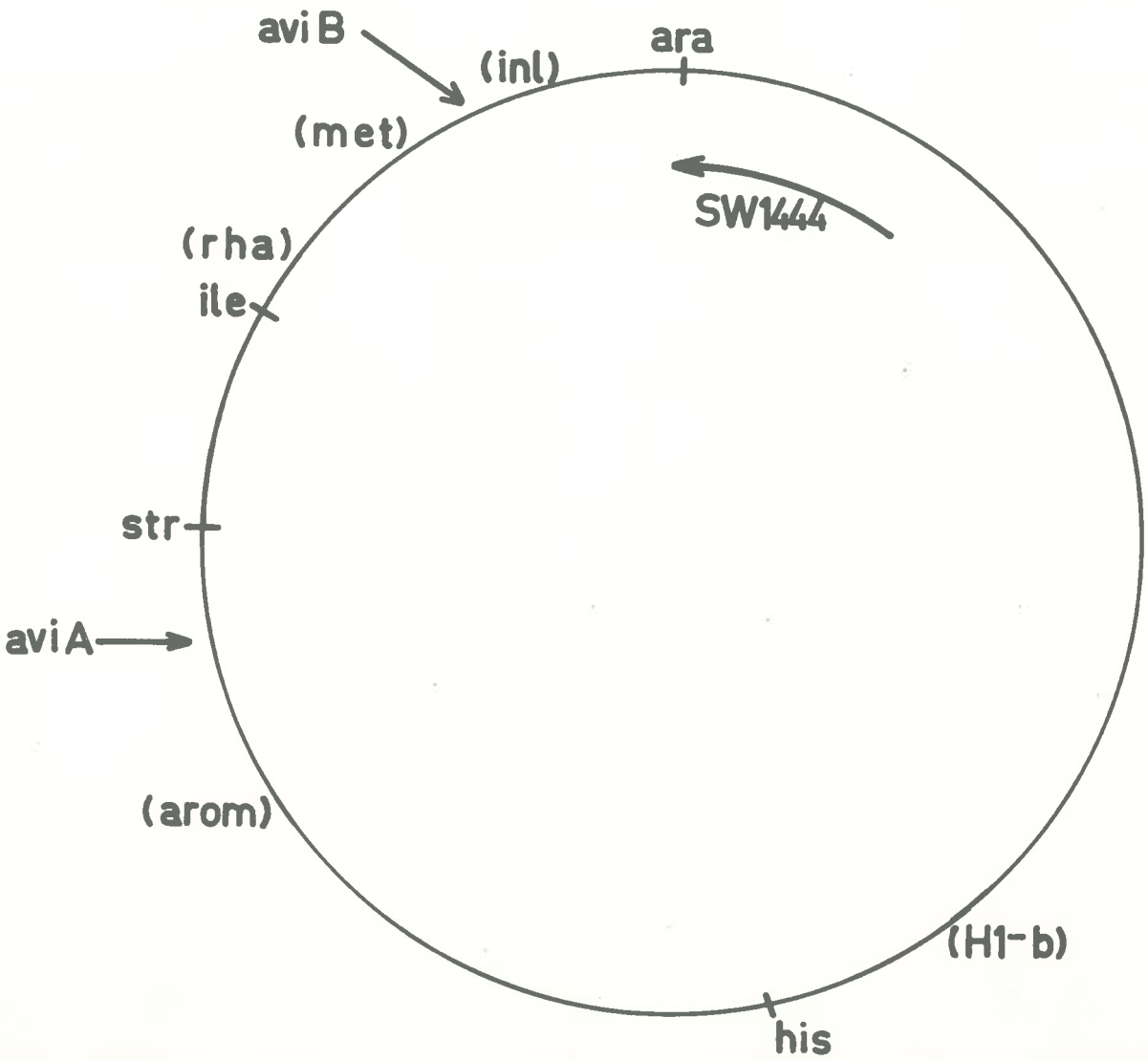


Different crossovers between the chromosomes will select for different recombinant types, which could lead to the segregation of the avirulence loci. The relative marker positions are not shown but all the genetic markers used in crosses between the S.abony Hfr and the S.typhimurium C5 recipients are indicated. For simplicity, only the crossovers required for the formation of partial, avirulent hybrids are depicted. The C5 chromosome is shown with ara- or ara+ since the C5 His- recipient was ara+ while the C5 His- Ara- recipient was ara-.

---- = crossovers; avi = avirulence loci; o___ = direction of gene transfer by the donor

FIGURE 11 THE SALMONELLA CHROMOSOME SHOWING THE LOCATION OF THE AVIRULENCE LOCI (*avrA* AND *avrB*) IN RELATION TO OTHER GENETIC MARKERS

For marker locations see text.


aviB
(inl)
ara
(met)
SW1444
(rha)
ile
str
aviA
(arom)
(H1-b)
his

16 that the hybrids which are partially avirulent, do not possess the completely avirulent character of the S.abony Hfr. Therefore, additional crosses were performed to determine if 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 initial hybrid C5 His+ Ara- Str-r (Table 15) possessing the LD50 = 1 x 10^5 bacteria , was backcrossed with the S.abony donor, with selection being made for the rha+ marker of the donor. Table 17 illustrates the three genetic classes of backcross hybrids and the LD50 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 to the initial partially avirulent hybrid. This was done so that decreases may be compared directly with those shown previously (Tables 14, 15 and 16). It is apparent that only the backcross hybrid which received the inl+ region, in addition to the initially transferred str region, is completely avirulent. In other words, the complete avirulence of this backcross hybrid is interpreted as being due to the fact that it received the respective S.abony alleles at both the aviA and aviB loci. The other two backcross hybrids are still partially avirulent since they failed to inherit the S.abony allele at the aviB locus. That

TABLE 17 VIRULENCE OF HYBRIDS FROM THE BACKCROSS: S.ABONY HFR SW1444 x S.TYPHIMURIUM C5 HIS+ ARA- STR-R (INITIAL HYBRID)

Bacterial strain	Hybrid classes		LD50 (bact)	Relative LD50[a]
	donor markers	recipient markers		
SW1444 (donor)	-	-	$1x10^8$	2,000,000
C5 His+Ara-Str-r (initial hybrid as recipient)	-	-	$1x10^5$	2,000
backcross hybrids	rha[b]	his ara inl H1 H2	$3x10^6$	60,000
	rha[b] inl H1	his ara H2	$1x10^8$	2,000,000
	rha[b] ara	his inl H1 H2	$8x10^5$	16,000

[a]Expressed as the ratio of the LD50 of the strain under consideration to that of the LD50 of strain C5 His-Ara-.

[b]Marker selected from donor.

the completely avirulent backcross hybrid of S.typhimurium C5 was not a chance isolation of a donor clone was indicated by the fact that it still retained the ara-, his+ and the H2-1,2 markers of the S.typhimurium initial hybrid recipient.

From these results it may be concluded that each avirulence locus by itself only specifies the phenotypic expression of partial avirulence in the hybrids. But both aviA and aviB are presumably necessary for the expression of complete avirulence.

Discussion and conclusions

68 hybrids, representing different genotypes arising from recombination between an avirulent S.abony donor and virulent S.typhimurium C5 recipients were examined for their mouse virulence. It was found that 15 (or 22 %) of these hybrids exhibited virulence which was intermediate between those of the S.abony and that of S.typhimurium. Two avirulence genetic loci (aviA and aviB) were postulated to be on the S.abony chromosome. The segregation of one or other of these loci during genetic recombination led to the phenotypic expression of intermediate virulence in the hybrids. When the S.abony alleles at both the loci were inherited by the hybrids, complete avirulence was expressed which was phenotypically indistinguishable from the avirulence of S.abony.

If the hybrids were segregating, partial heterozygotes with a frequency of segregation of recipient alleles below the detectable level (<0.5 %; see last Chapter) then on challenge into mice the segregation of the S.typhimurium C5 alleles at the aviA and aviB loci should lower the LD50 values of the hybrids. For example, if the completely avirulent backcross hybrid (Table 17) possessing the observed LD50 value of 1×10^{8} bacteria was a heterozygote, then even if segregation occurred at a frequency of 10^{-3} bacteria or less, the proportion of C5 organisms will rapidly come to predominate in the mouse. This would obviously lead to the death of the animal. Reconstruction experiments performed by Furness and Rowley (1956) showed that mice challenged intraperitoneally with 10 cells of virulent S.typhimurium C5 and 10^{7} cells of avirulent S.typhimurium (a ratio of $1 : 10^{6}$) were found to increase the ratio to 1 : 9 by the 5th day post-challenge. In fact, the backcross hybrid was as avirulent as the S.abony donor. Therefore, allelic substitution at the aviA and aviB loci could be assumed to have occurred. It means then that the difference in mouse virulence between S.typhimurium strain C5 and S.abony is controlled by two genetic loci.

The aviA locus was found to map close to the str locus and the aviB locus close to the inl locus. The avirulence loci are therefore separated by about 25 % of the bacterial

chromosome. Hybrids inheriting the S.abony allele at the aviA locus show a scatter of LD50 values between $1 \times 10^5 - 2 \times 10^7$ bacteria with an average LD50 = 3×10^6 bacteria. The hybrids inheriting the S.abony allele at the aviB locus do not show this spread (LD50 = $1\text{-}2 \times 10^5$ bacteria) although only a small number of such hybrids were studied . The significance of the spread in the partial avirulence pattern of the hybrids inheriting the donor allele at the aviA locus is not entirely clear in genetic terms. It may be that sites within this locus specify the quantitiative aspects of avirulence as measured in terms of the LD50.

Chapter 8

GENETICS OF PARTIAL VIRULENCE IN CROSSES BETWEEN S.TYPHIMURIUM HFR SR305 OR HFR SR305 GAL+ AS DONOR AND S.TYPHIMURIUM SW1292 AS RECIPIENT

The difference in mouse virulence between S.typhimurium strain C5 and S.abony was found to be controlled by two genes (Chapter 7). This was established indirectly by analysing the genetics of avirulence in recombination experiments between the avirulent S.abony Hfr SW1444 and virulent S.typhimurium C5 recipients. A direct study of virulence was not possible using fully virulent-donor and avirulent-recipient combinations since a suitable recombination system was not available (Chapter 5). Alternatively, the genetics of partial virulence was studied since it was found that S.typhimurium Hfr SR305 recombined with S.typhimurium SW1292. The LD50 values of SR305 and SW1292 are 5×10^4 bacteria and 1×10^7 bacteria, respectively (Table 7; Chapter 5). The difference in LD50 levels between these two strains is 200 fold. SR305 is referred to as being partially virulent since its LD50 value is intermediate between that of the LD50 levels of fully virulent and completely avirulent strains (Table 7; Chapter 5).

Recombination pattern between Hfr SR305 Gal+ and SW1292

A gal+ mutant of SR305 was isolated (see Materials

and Methods) since gal was an extra marker that could be used in genetic studies with SW1292. Table 18 shows the frequency of transmission of markers from Hfr SR305 Gal+ into SW1292. (Pro+ leu+) was a joint selection since each marker separately had a reversion frequency to prototrophy which was close to the detectable recombination frequency for prototrophic recombinants. A similar frequency of transmission of markers was also observed when the donor was Hfr SR305 which was the parental strain of Hfr SR305 Gal+. The frequency of recombination in this system was low and xyl+ recombinants were not detected under the conditions of mating. However, the rha+, mal+ and ara+ recombinants were analysed for their content of unselected markers from the donor. The prototrophic (pro+ leu+) and gal+ recombinants were not analysed since these recombinants were few in numbers. Table 19 shows the extent of linked transfer of genes from the Hfr SR305 Gal+ donor into the recipient. The first 3 markers transferred by the Hfr donor are rha+, mal+ and ara+ whose order of transfer cannot be distinguished on the basis of the frequencies of recombination shown in Table 18. However, the transfer sequence appears to be rha--- leu--- pro---gal---xyl. The results show that the majority of hybrids receive the donor markers rha+, mal+, ara+ and (pro+leu+). Fig.12 shows the location of the different

TABLE 18 FREQUENCY OF TRANSMISSION OF MARKERS FROM S.TYPHIMURIUM HFR SR305 GAL+ TO S.TYPHIMURIUM SW1292 (RECIPIENT)

Supplements added to minimal selective medium	Selected marker	Frequency per donor cell[a]
rhamnose[b], proline, leucine and streptomycin	rha+	5×10^{-7}
maltose[b], proline, leucine and streptomycin	mal+	5×10^{-7}
arabinose[b], proline, leucine and streptomycin	ara+	5×10^{-7}
glucose[b]	(pro+leu+)	2×10^{-8}
galactose[b], proline, leucine and streptomycin	gal+	1×10^{-9}
xylose[b], proline, leucine and streptomycin	xyl+	$<1 \times 10^{-9}$

The Hfr contraselecting markers were his- and str-s. The frequencies refer to the approximate number of recombinants obtained by the standard mating procedure.

[a] Frequency of recombination

[b] Carbon source

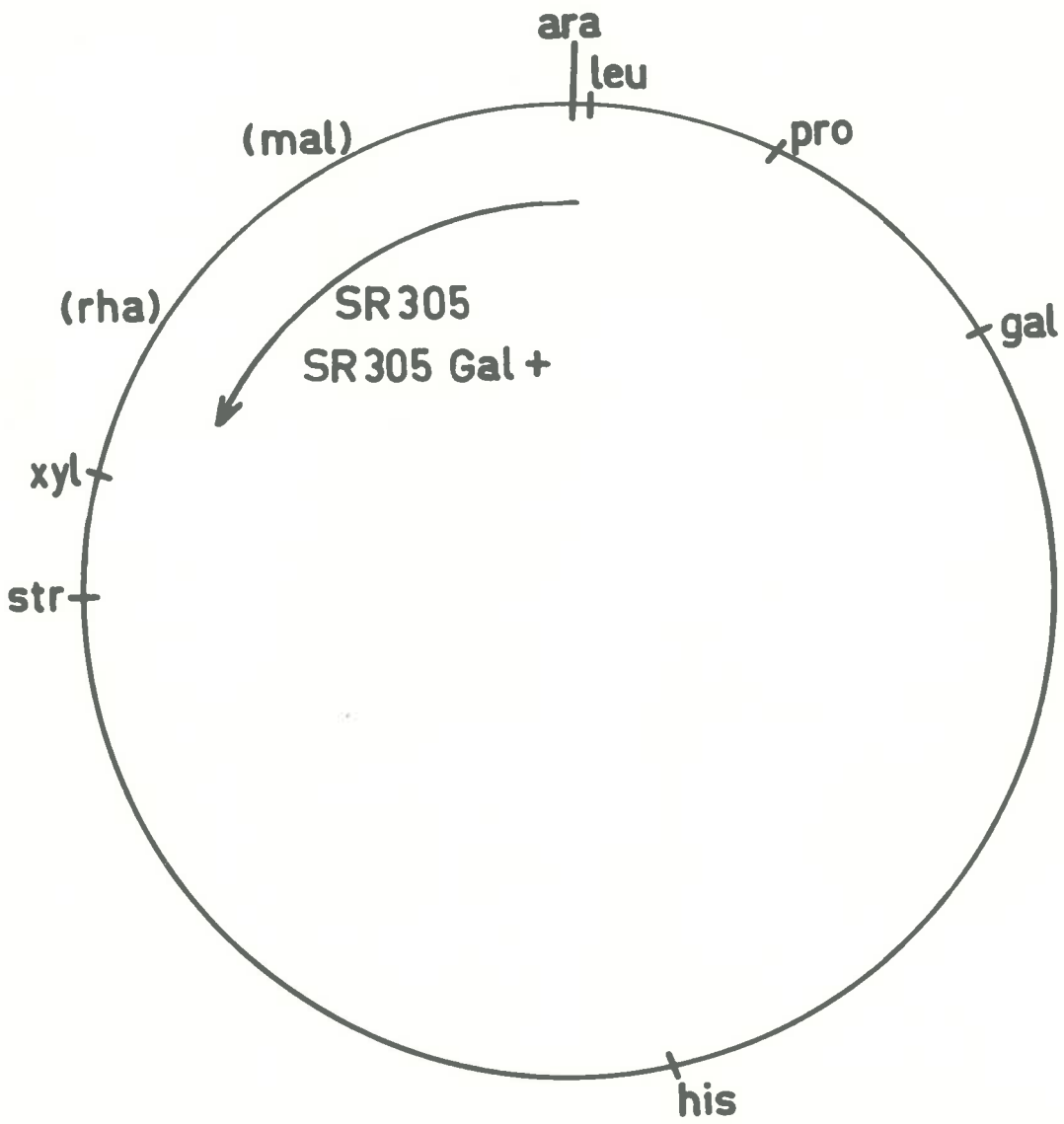
TABLE 19 LINKAGE OF UNSELECTED MARKERS IN CROSSES BETWEEN S.TYPHIMURIUM HFR SR305 GAL+ AND S.TYPHIMURIUM SW1292 (RECIPIENT)

Selected marker	Percentage frequency of unselected markers from donor				
	rha+	mal+	ara+	(pro+leu+)	gal+
rha+	-	65	65	67	1
mal+	50	-	32	54	17
ara+	88	98	-	96	4

One hundred rha+ and fifty each of mal+ and ara+ recombinants were analysed for the unselected markers shown.

FIGURE 12 THE CHROMOSOME MAP OF SALMONELLA

It shows the location of the genetic markers used in recombination experiments between S.typhimurium Hfr strains SR305 or SR305 Gal+ as donor and S.typhimurium strain SW1292 as recipient. The loci in parentheses indicate that the location of the marker in relation to the neighbouring markers has not been determined precisely. The inner line refers to the order of transfer of markers by the Hfr donors.


ara
leu
pro
gal
his
str
xyl
(rha)
(mal)
SR305
SR305 Gal +

markers used in these crosses. It was constructed on the basis of the E.coli K-12 map (Jacob and Wollman, 1961), and it is used primarily for the discussion relating to the genetics of partial virulence.

Virulence of hybrids from crosses using either S.typhimurium Hfr SR305 or Hfr SR305 Gal+ as donor and S.typhimurium SW1292 as recipient

Observations on mouse virulence titrations

Before the hybrids are examined for virulence, certain aspects of the virulence of the strains being studied are worthy of consideration since these have relevance later on. They are easily seen by an examination of Table 20. It shows the pattern of killing produced in mice challenged with the donor (SR305), the recipient (SW1292) and two hybrids for comparative purposes. The cumulative numbers of the mice dying is shown till the 15th day post-challenge and also the numbers dead by the 28th day (end of experiment). A striking feature apparent from the Table is that mortality in mice challenged with SR305 is progressive with time, commencing 3 to 4 days after challenge. In addition mortality is dose dependent. In contrast, 80 % to 90 % mortality occurs within 48 hours of challenge with 2.5×10^7 bacteria of strain SW1292. No further deaths occur after 2 days. A three-fold reduction in the dose produces only 10 % mortality. In other words, SW1292 causes an endo-

TABLE 20 CUMULATIVE NUMBERS OF MICE DYING AFTER CHALLENGE WITH S.TYPHIMURIUM DONOR, RECIPIENT OR HYBRID STRAINS

Bacterial strain	Days after challenge 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15 // 28	Dose (bact/mouse)	LD50 (bact)
SR305 (donor)	0	0	0	8	9	9	9	9	9	9	9	9	9	9	10	9.1×10^{6}	7×10^{4}
	0	0	0	4	6	8	8	8	8	8	8	8	8	8	8	9.1×10^{5}	
	0	0	0	0	1	1	3	3	5	5	5	5	5	5	5	9.1×10^{4}	
	0	0	0	0	1	1	2	2	2	2	2	2	2	2	3	9.1×10^{3}	
SW1292 (recipient)	8	9	9	9	9	9	9	9	9	9	9	9	9	9	9	2.5×10^{7}	1×10^{7}
	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	7.5×10^{6}	
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2.5×10^{6}	
hybrid[a] xyl+mal+	0	0	0	3	3	3	8	8	10	10	10	10	10	10	10	4.0×10^{6}	4×10^{5}
	0	0	0	0	0	0	2	2	4	4	4	4	4	5	5	4.0×10^{5}	
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4.0×10^{4}	
hybrid[a] rha+ara+(pro+leu+)	0	0	5	5	5	5	7	7	7	7	7	7	7	7	8	4.0×10^{6}	2×10^{6}
	0	0	2	2	2	2	3	3	3	3	4	4	4	4	4	2.0×10^{6}	
	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	2.0×10^{5}	

The number of mice challenged with each dose level of bacteria is 10

[a] The donor markers inherited by the hybrid are shown

toxic type of death with absence of dose response mortality when a high dose (2.5×10^7 bacteria) is used, otherwise it is unable to multiply or produce infections. SW1292 is thus similar to S.abony Hfr SW1444 in the manner in which mice are killed (Chapter 7). Therefore, Hfr SR305 is distinguishable from SW1292 not only by LD50 values but also by the type of death that is produced in groups of infected animals. Hfr SR305 causes an infective type of death whilst SW1292 causes an endotoxic type. The LD50 difference and the type of death produced in mice will be used as two separate phenotypic attributes of virulence in the study of the hybrids. Now if the two hybrids shown in Table 20 are examined they display the infective characteristic of the donor. The LD50 of SR305 is shown in the Table to be 7×10^4 bacteria whereas at other times in this thesis it is referred to as having a LD50 = 5×10^4. This is because the former was estimated from a single titration experiment but the latter is the average LD50 value from a number of experiments.

Virulence of hybrids

Different classes of genetic hybrids from crosses between SR305 Gal+ and SW1292 (Table 19) and hybrids from crosses between SR305 and SW1292 were examined for their mouse virulence. The hybrids possessed different combinations of donor markers from the chromosomal segment

rha--- gal (Fig.12). In addition, hybrids possessing the last marker (xyl+) together with proximal markers transferred by Hfr SR305 were also tested. 6 such xyl+ hybrids were selected and purified by Dr.L.S.Baron. They were selected when the standard mating procedure utilized a much larger number of recipients ($>5 \times 10^9$ cells) than that used here when xyl+ recombinants were not detected (Table 18). The results of the virulence titration of hybrids are documented in Tables 21 and 22. In these Tables the relative LD50 is expressed as the ratio of the LD50 of the recipient (SW1292) to that of the LD50 of the hybrid. This is used as the measure of the increase in virulence of hybrids in terms of LD50 values (note that in Chapter 7 the relative LD50 is used as a measure of the decrease in virulence of hybrids). The final columns in Tables 21 and 22 indicate the type of death produced in mice by the various hybrids. The infective or endotoxic type of the hybrids is based on the distinction discussed in the previous section.

In terms of virulence as measured by LD50 values, two of the three hybrids possessing the donor markers xyl+ mal+ have each a LD50 = 4×10^5 bacteria (Table 21). Similarly, one of the three hybrids possessing the donor markers gal+ rha+ has a LD50 = 4×10^5 bacteria (Table 22). The increase in virulence in relation to the SW1292 recipient

TABLE 21 VIRULENCE OF HYBRIDS FROM CROSSES BETWEEN S.TYPHIMURIUM STRAINS HFR SR305 AND SW1292

Bacterial strain	Hybrid classes: donor markers	Hybrid classes: recipient markers	No. tested	LD50 (bact)	Relative LD50[a]	Type of death[b]
SR305 (donor)	-	-	-	$5x10^4$	200	infective
SW1292 (recipient)	-	-	-	$1x10^7$	1	endotoxic
hybrids	rha[c] mal ara (pro leu)	xyl str	1	$6x10^6$	1-2	infective
	rha[c] ara (pro leu)	mal xyl str	1	$2x10^6$	5	"
	rha[c] mal (pro leu)	ara xyl str	1	$2x10^6$	5	"
	rha[c] mal	ara xyl (pro leu) str	1	$>6x10^6$	1	no deaths
	rha[c]	mal ara xyl(pro leu)str	6	$2-3x10^6$	3-5	infective
	xyl[c] rha mal	ara (pro leu) str	2	$2x10^6$	5	"
	xyl[c] mal	rha ara (pro leu)str	1	$2x10^6$	5	"
	xyl[c] ara (pro leu)	rha mal str	1	$3x10^6$	3-4	"
	xyl[c] mal	rha ara (pro leu) str	2	$4x10^5$	25	"

[a] Expressed as the ratio of the LD50 of SW1292 to that of the LD50 of the strain under consideration.

[b] See text for type of death produced in mice.

[c] Marker selected from donor.

TABLE 22 VIRULENCE OF HYBRIDS FROM CROSSES BETWEEN S.TYPHIMURIUM STRAINS HFR SR305 GAL+ AND SW1292

Bacterial strain	Hybrid classes: donor markers	Hybrid classes: recipient markers	No. tested	LD50 (bact)	Relative LD50[a]	Type of death[b]
SR305 Gal+ (donor)	-	-	-	$1x10^5$	100	infective
SW1292 (recipient)	-	-	-	$1x10^7$	1	endotoxic
hybrids	gal[c] ara(pro leu)	rha mal xyl str	1	$6x10^6$	1	infective
	gal[c] rha	ara mal xyl (pro leu)str	2	$4-5x10^6$	2-3	"
	gal[c] rha	ara mal xyl (pro leu)str	1	$4x10^5$	25	"

[a] Expressed as the ratio of the LD50 of SW1292 to that of the LD50 of the strain under consideration.

[b] See text for type of death produced in mice

[c] Marker selected from donor.

is 25 fold but the LD50 level is intermediate between that of the donor strains and that of the recipient. All the other hybrids have LD50 values which are not markedly different from that of the recipient (increases in virulence between 1-5 fold).

However, if the type of death produced in mice is examined, all the hybrids except the one rha+ mal+ hybrid (19/20) produced an infective type similar to the donor SR305 or SR305 Gal+. The pattern of killing of challenged animals is shown only for two hybrids (Table 20) but 19 of the 20 hybrids mentioned in Tables 21 and 22 behaved similarly to the two hybrids shown in Table 20. In this Table, it can be seen that even though the two hybrids have different LD50 values, they both still produce infective rather than endotoxic deaths in mice. That the infective characteristic of hybrids was due to recombination (whereby they inherited the characteristic from the donor) was indicated by the following finding. 10 single-colony isolates from nutrient agar of the recipient SW1292 were prepared individually for challenge into groups of 10 mice. The inoculum varied between 5×10^6 - 1×10^7 bacteria/mouse. 21 days post-challenge, either 1 or 0 mouse died in each challenge group. On the contrary, with the same challenge dose, 6- 10 mice died when challenged with the hybrids. In other words, the infective characteristic displayed by the

hybrids was not due to extreme variability within the cell population of the recipient.

Discussion

2 hybrids inheriting the donor markers xyl+ mal+ (separated by the origin) acquired a 25 fold increase in virulence in terms of LD50 values. A similar increase was observed with 1 hybrid inheriting the donor markers gal+ rha+. The hybrids inheriting xyl+ mal+ from the donor were selected for the last marker (xyl+) transferred by Hfr SR305 (Fig.12). These hybrids received as unselected markers either rha+ mal+, mal+ alone or ara+ (pro+leu+), (Table 21). These unselected markers are from the early part of chromosome transferred by this Hfr. The hybrids inheriting xyl+ as the selected marker from the donor randomly receive proximal chromosomal material from the Hfr SR305. Therefore, it is not possible to determine to what locus or loci the partial virulence loci of the Hfr may be adjacent, whose transfer confers upon the hybrids virulence intermediate between that of the donor and the recipient. Similarly, with the hybrids inheriting the donor markers gal+ rha+ (Table 22). These hybrids received from Hfr SR305 Gal+ the lead marker rha+ as the unselected marker but did not receive the markers mal+, ara+ and (pro+ leu+) which are situated in between rha and gal (Fig. 12). Because of the random nature of the chromosomal pieces that

were detected in the hybrids no definite conclusions may be drawn as to the location of the loci involved in the control of partial virulence.

However, if the aviA and aviB loci that have been postulated to control the difference in mouse virulence between S.abony and S.typhimurium C5 (see last Chapter) could be used to explain the findings discussed here, then it is probable that the recombinants inheriting the donor markers xyl+ mal+ increased in their virulence because they inherited the S.typhimurium Hfr SR305 allele at the aviB locus. If the chromosome maps shown in Figs. 11 and 12 are comparable then it is possible that the mal+ marker is very close to aviB, shown in Fig.11. It is of interest that the LD50 value of the hybrids receiving the donor markers xyl+ mal+ (LD50 = 4 x 10^5 bacteria) was intermediate between that of the S.typhimurium SR305 donor and that of the S.typhimurium SW1292 recipient (Table 21). In other words, the lack of expression of the LD50 characteristic of the donors (LD50 = 5 - 10 x 10^4 bacteria) may be due to the lack of inheritance of the donor allele at the aviA locus shown in Fig.11. By analogy with the S.abony x S.typhimurium crosses (see last Chapter), the phenotypic expression in the hybrids of the LD50 value characteristic of the S.typhimurium donors would require the presence of the donor alleles at the aviA and aviB loci.

However, with the data presented in Tables 21 and 22, it is not possible to say whether the hybrids of intermediate virulence, inheriting the donor markers xyl+ mal+ or the markers rha+ gal+, inherited the donor allele at the aviA or aviB locus.

An interesting outcome of these studies was the finding that the infective characteristic of the S.typhimurium donors was inherited by nearly all the 20 hybrids tested although in terms of LD50 values only 3 hybrids showed an increased virulence in comparison to the recipient. There was no discernible correlation between the infective characteristic and any of the genetic loci whose segregation in the hybrids was studied. For example, even the 6 hybrids inheriting the donor marker rha+ alone have the infective characteristic of the donor (Table 21). In terms of the mechanism of killing of mice it would seem that what the hybrids inherit from the donor is the ability to multiply to a limited extent. In Table 20, it can be seen that with a challenge dose of 7.5×10^6 bacteria of SW1292 only 1/10 mice dies. But with 4.0×10^6 bacteria of the hybrid possessing a LD50 = 2×10^6 bacteria, 8/10 mice die by the end of the experiment. The fact that nearly all the hybrids (19/20) inherit the infective characteristic of the donor may mean that the genetic locus or loci determining this characteristic is associated with a plasmid

(lederberg, 1952; Clark and Adelberg, 1962). For example, it may be similar to the high frequency of transfer of lac+ by the sex factor (F) in strain K-12 of E.coli (Jacob and Wollman, 1961). Further experimental work is necessary to test the validity of this hypothesis.

Conclusions

3 out of 20 hybrids from crosses between partially virulent donor strains of S.typhimurium (LD50 = 5 = 10 x 10^4 bacteria) and an avirulent recipient (LD50 = 1 x 10^7 bacteria) were found to possess intermediate virulence (LD50 = 4 x 10^5 bacteria). The genetic loci specifying this increased virulence (as measured in terms of LD50 values) was not mapped because of the difficulty in interpreting linkage data. In addition, it was found that nearly all the 20 hybrids inherited the infective characteristic of the donors. It was postulated that the locus or loci determining the phenotypic expression of this property may be associated with a plasmid.

Chapter 9

DISCUSSION

This thesis is concerned with studies on the genetic control of virulence of *S.typhimurium* for mice. Different strains of Salmonella were analysed with the view to establishing whether the bacterial property of virulence has a genetic basis. After initial failures to develop a suitable recombination system between virulent and avirulent strains, an avirulent *S.abony* donor was found to readily recombine sexually with the virulent strain C5 of *S.typhimurium* as recipient. By genetic recombination different segments of the *S.abony* chromosome were transferred into the virulent C5 strain. The virulence property was studied as an unselected marker in the hybrids. It was found that two loci - aviA and aviB controlled the difference in virulence between *S.typhimurium* strain C5 and *S.abony*.

When *S.typhimurium* C5 Leu- was transduced to prototrophy and the prototrophic transductants subsequently examined for their virulence, it was found that their virulence was still similar to C5 Leu- (Table 3). This may be explained in genetic terms by the finding of the aviA and aviB loci being responsible for the virulence difference between *S. typhimurium* strain C5 and *S.abony*.

The LD50 value of C5 Leu- is approximately equivalent to the LD50 values of the partially avirulent hybrids inheriting the S.abony allele at the aviA or the aviB locus (Chapter 7). Therefore, the avirulence of C5 Leu- could be due to mutation to partial avirulence either at the aviA or the aviB locus in the parental virulent C5 wild type strain. The leu marker is very close to the right of the ara marker shown in Fig. 11. Therefore, by performing transduction to prototrophy of strain C5 Leu-, it is unlikely that the C5 wild type allele at the aviA or aviB locus would have been cotransduced with the leu+ marker by phage PLT-22. Even the cotransduction of the C5 wild type allele at the aviB locus and the leu+ marker would not have been possible since these are separated by about 4-5 % of the bacterial chromosome and hence their simultaneous transduction by phage PLT-22 would be rare and consequently undetectable.

The above approach to study the genetics of virulence by analysing the bacterial chromosome by appropriate recombination techniques has only been explored in the past in a limited number of systems. Attempts to directly transfer virulence to the avirulent S.typhimurium strain M206 (LD50 = 9×10^6 bacteria) by phage PLT-22 lysates obtained from the virulent S.typhimurium strain C5 (LD50 = 14 bacteria) were unsuccessful (Furness and Rowley, 1956). Since

M206 was prototrophic, an *in vitro* selection procedure could not be used to select virulent transductants. Therefore, they used the mouse as the selective "medium" for the enrichment of virulent M206 transductants that may be obtained. Strain M206 was originally derived by Jensen (1929) from the virulent *S.typhimurium* strain Br1 , by a series of *in vitro* passages at increasing growth-temperature. If the aviA and aviB loci established in the genetic studies of *S.abony* x *S.typhimurium* C5 crosses were assumed to be generally responsible for the mouse virulence or avirulence of Salmonella, then it is likely that M206 was a mutant derivative of Br1 which could have had a mutation to avirulence at the aviA, aviB or at both of these loci consequently. The failure to transduce full virulence to M206 (Furness and Rowley, 1956) may have been due to a low frequency of transduction ($<10^{-6}$ of treated cells) at the aviA or the aviB locus. Since samples containing about 10^6 treated avirulent organisms were examined, any virulent transductants present would not have been detected. Or, by analogy with the *S.abony* x *S.typhimurium* C5 system where complete avirulence required the presence of *S.abony* alleles at both the aviA and aviB loci, the expression of full virulence would require the simultaneous transduction of both alleles at aviA and aviB of strain C5 into strain M206. Since transductants were only examined for full

virulence it was unlikely that both the C5 alleles would have been transduced simultaneously, as aviA and aviB are separated by about 25 % of the bacterial chromosome. Loci separated by this distance have never been reported to be cotransducible with phage PLT-22. In the studies of Furness and Rowley (1956) it was not determined whether an intermediate level of virulence could be transduced from strain C5 to strain M206.

While studying the instability of hybrids formed between an E.coli K-12 Hfr and S.typhosa 643 Vi-positive recipient, Baron et al., (1960) observed that a relatively stable partial heterozygous hybrid (X30D) was of intermediate mouse virulence. The donor had a LD50 =(37 $\pm$ 10) x 10^8 bacteria, while the recipient's LD50 was (25 $\pm$ 10) x 10^6 bacteria. The heterozygote was later shown by Falkow et al., (1962) to segregate haploid bacteria in which one type had incorporated the entire coli transferred material from the marker for phage T6 sensitivity to the marker for xylose-utilisation (X30P). The other type had incorporated a similar length of donor material except by recombination about the middle of the transferred piece, it still retained the parental Salmonella material from the marker for phage T1 resistance to the marker for arginine + uracil (X30T). This Salmonella chromosomal piece most

likely includes the aviB locus studied in this thesis (see Fig.11; Jacob and Wollman, 1961). If so, the intermediate virulence of X30D observed by Baron et al., (1960) may have been due to the inheritance of the E.coli K-12 allele at the aviB locus. It is of interest that the LD50 of X30F was almost identical to that of X30D whereas that of X30T was similar to that of the S.typhosa 643 Vi-positive parent. These results could be explained by the assumption that X30T lost the E.coli allele at the aviB locus by segregation but that it was integrated in X30F. It may also be speculated that the E.coli allele at the aviB locus was dominant to the allele in S.typhosa 643 Vi-positive since X30D was of intermediate virulence but it segregated X30T which exhibited virulence, characteristic of S.typhosa 643 Vi-positive while the virulence of X30F was identical to that of X30D. The lack of inheritance of the complete avirulence characteristic of the E.coli donor might have been due to the lack of inheritance of the E.coli allele at aviA in addition to that at aviB which, by analogy with the S.abony x S.typhimurium C5 system, would be necessary for the expression of complete avirulence. This appears likely since aviA, which is close to str, was outside the chromosome region studied by Baron et al., (1960).

Falkow et al., (1963) have studied the genetics of virulence of hybrids arising from crosses between S.flexneri strain 2a and E.coli K-12 donors. The host animal was the pretreated guinea pig (either starved or carbon tetrachloride treated) challenged orally with a single dose of 5-10 x 10^7 bacteria. In such challenged animals the Shigella recipient produced about 80 % mortality while the E.coli donors produced about 5 % mortality. On this basis, the Shigella was deemed virulent and the E.coli avirulent. The "all or none" type of response was used to assess the virulence of hybrids. They found that some hybrids receiving the chromosomal region rha--- mal (note this mal locus is closely linked to str and it is distinct from the mal locus studied in this thesis; Fig. 12), from the avirulent donor became completely avirulent. Interestingly enough, this region is very close to or includes the aviA locus, which is one of the two loci controlling the difference in virulence between S.typhimurium strain C5 and S.abony (Fig.11). Falkow et al., (1963) also found that Shigella hybrids inheriting the ability to utilise the carbohydrate fucose (fuc+) from E.coli donors, had an intermediate virulence (about 30 % mortality in infected guinea pigs). Fuc+ has been mapped near the str locus (Falkow, 1963). If this is correct, then the intermediate virulence of the Shigella hybrids could be due to the

inheritance of the E.coli allele at the aviA locus (Fig.11). One other chromosomal region was associated with the expression of intermediate virulence in Shigella hybrids. This was the region ara---pili. The locus for the synthesis of pili (appendages on the bacterial cell surface) has been mapped by Brinton et al., (1961). This locus is very close to the inl marker shown in Fig.11. Therefore, the intermediate virulence of the Shigella hybrids inheriting the ara---pili region from E.coli could be due to the inheritance of the E.coli allele at the aviB locus shown in Fig.11. Shigella hybrids receiving other chromosomal regions from E.coli donors were found to be similar in their virulence to the parent Shigella recipient (Falkow et al., 1963). From a study of segregating, partial heterozygotes in which the diploid region encompassed the rha---mal region, they found that such diploids were of intermediate virulence. However, haploid segregants which had lost the E.coli genetic material expressed the full virulence characteristic of the original S.flexneri strain 2a. A single haploid segregant which had integrated the coli segment rha---mal, was as avirulent as the E.coli donors. This suggested that avirulence was dominant to virulence in Shigella. The overall similarity in the findings of Falkow et al., (1963) in E.coli x Shigella crosses and those found here

to control the difference in virulence between S.typhimruium strain C5 and S.abony is all the more amazing since entirely different host-parasite systems were studied.

Having shown that aviA and aviB control the difference in virulence between S.typhimurium C5 and S.abony it would have been interesting to determine what the nature of the changes are in relation to virulence. Some idea of this might have been gained if virulence was determined to be dominant or recessive to avirulence in the crosses between S.abony Hfr SW1444 and S.typhimurium C5 recipients. This would have been possible by an analysis of segregating, partial heterozygotes. Unfortunately heterozygotes were not detected amongst the hybrids from crosses between S.abony Hfr and S.typhimurium C5. However, as has been discussed previously, there is some evidence that the avirulence allele is dominant to that for virulence (Baron et al., 1960; Falkow et al., 1963). In the absence of any evidence as to whether the avirulence or virulence allele is dominant at the aviA and aviB loci, it is not possible to state what the biochemical basis is likely to be for the partial avirulence of the hybrids from crosses between S.abony and S.typhimurium C5. It may be that the S.abony alleles at the aviA and aviB loci 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 strain C5.

The avirulence of Salmonella strains for the mouse has sometimes been shown to be due to the requirement for various kinds of growth factors. For example, Bacon et al., (1951) found that loss of virulence of S.typhosa strains was associated with a strain's requirement for purine, para-amino benzoic acid or aspartic acid. These growth factors were shown to be in limiting concentrations in the peritoneal fluids of the mouse. The S.typhosa strains were, however, restorable to their original virulence either by prototrophic reversion or by providing the specific growth factor simultaneously with challenge inocula. In other words, the avirulence of the S.typhosa strains was due to inability to multiply in mice because of growth factor limitations. A similar situation was encountered by Furness and Rowley (1956) in the avirulence of the ade- strains D2 and D10 of S.typhimurium . These strains could be restored to full virulence by transduction to prototrophy in vitro with phage PLT-22.

Therefore, it was natural to determine whether avirulence was associated with growth-factor requirements in the studies reported in this thesis. The leucine-requiring mutant C5 Leu- of S.typhimurium was found to be unaltered in its relative avirulence even after transduction

to prototrophy (Table 3). All the avirulent hybrids obtained from the crosses between S.abony Hfr and S.typhimurium C5 (Tables 14, 15, 16 and 17) were prototrophic except the inl+ hybrids (Table 14) 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 for growth were virulent (Tables 14 and 15). Similarly, with the hybrids arising from crosses between S.typhimurium Hfr SR305 or Hfr SR305 Gal+ and S.typhimurium SW1292 (Chapter 6). The avirulence of SW1292 is not due to its growth requirement for proline and leucine since the 3 hybrids showing an increased virulence in terms of LD50 values (Tables 21 and 22) still require these factors for growth. In addition, several prototrophic hybrids were still as avirulent as SW1292 when LD50 values are compared (Tables 21 and 22). There is also no correlation between the type of death produced in mice and a strain's requirement or non-requirement for these growth factors (Tables 21 and 22). Therefore, the avirulence of the many different strains that have been studied here are not necessarily associated with a particular strain's growth requirement(s).

It is generally believed that smooth to rough variation is associated with either complete or partial loss of virulence of bacteria (Wilson and Miles, 1955).

However, all the Salmonella strains used here, whether virulent or avirulent, were smooth in colonial appearance on solid medium.

Finally, the comparative aspects of the genetics of virulence in different groups of enteric bacteria were discussed because it has been shown that chromosomal gene order and distance between markers are identical in E.coli K-12, Salmonella and Shigella (see literature review in Chapter 2; Falkow et al., 1963; Schneider et al., 1963). In addition, the two-loci control of the difference in virulence between S.typhimurium strain C5 and S.abony has been extended to explain the genetic changes in virulence observed by previous workers. Moreover, there was general similarity in the chromosomal regions associated with virulence in S.flexneri strain 2a and S.typhimurium strain C5. In so discussing the assumption was made that virulence of the enteric bacteria has a common genetic basis. This assumption may be unwarranted. For example, when more extensive mapping of bacterial chromosomes in these bacteria (especially in the Salmonella and the Shigella) is performed it is possible that gene loci controlling the property of virulence are quite distinct in different groups of enteric bacteria. Further such loci may turn out to be quite complex and comprise a number of mutational sites each responsible for different attributes of virulence

or avirulence in different groups of enteric bacteria. At the present time what lends credence to the validity of the assumption is that there is gross similarity in the genetic interpretation of the control of virulence amongst these bacteria. Therefore, more extensive work is needed with many different experimental systems to determine the possible significance of this concurrence.

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