GENETIC STUDIES IN SALMONELLA PULLORUM USING F-TRP, FT71, AND F-LAC DONORS

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ABSTRACT

GENETIC STUDIES IN SALMONELLA PULLORUM
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By

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The cysteine biosynthetic pathway in Salmonella pullorum was reported to be similar to that in S. typhimurium but unusual with respect to some biochemical properties. The primary purpose of this study was to compare the spacial relationship of the cysteine genes in S. pullorum to that reported for S. typhimurium to determine if any unusual genetic placement of the genes accompanied the unusual biochemical properties. During the course of this investigation, the donor properties of several S. pullorum donor strains were studied.

The mutants employed in this study were either naturally occurring or produced by N'-methyl-N'-nitro-N-nitrosoguanidine mutagenesis. These mutants were classified by auxanographic tests. The map location for genetic markers was determined by analysis of recombination frequency, interrupted mating, and unselected marker data on recombinants produced by conjugal transfer of the chromosome. Bacterial matings for genetic analysis

were performed by employing either the Millipore or broth mating techniques.

The S. pullorum F-trp donor was characterized by analysis of recombinants for inheritance of Trp^+ and MS2 phage sensitivity and by the curability of its F-trp using ethidium bromide.

The S. pullorum F-lac donors were characterized by analysis of recombination frequency, interrupted mating, and unselected marker data following broth matings with S. pullorum recipients. The curability of F-lac was tested using ethidium bromide treatment.

The cysteine mutants of S. pullorum were classified into four groups. Genetic analysis of recombinants produced when mutants representative of these groups were mated indicated that S. pullorum has its cysteine genes scattered around the chromosome rather than in a single locus or closely spaced loci.

The fact that Trp^+ in the F-trp S. pullorum donor (MS810) was not curable, and that the Trp^+ recombinants from MS810 matings were generally MS2 resistant suggest that this donor has the F-trp rather stably integrated into the chromosome to produce an Hfr type donor.

The two Lac^+ MS2 phage sensitive isolates selected following a mating between an E. coli F-lac strain and a pili-less, MS2 phage resistant ioslate of MS810 showed donor ability in matings with S. pullorum recipients.

One isolate donated its chromosome as the progenitor, MS810; the other isolate produced a high-frequency random transfer of the chromosome.

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INTRODUCTION

Genetic maps are an aid to understanding the control of biosynthetic pathways in bacteria. In the bacteria, Escherichia coli and Salmonella typhimurium, must is known about both the control of amino acid biocynthetic pathways and the location on the genetic map of the genes necessary for the synthesis of the enzymes essential to the pathway. The pathway of particular interest to us in our laboratory is that of cysteine biocynthesis since Salmonella pullorum wild type in our laboratory was found to be a double cysteine auxotroph. This S. pullorum auxotroph could be reverted to cysteine prototrophy (62).

When the cystein biosynthetic pathway of S. pullorum was compared to that of S. typhimurium, it was found to be quite similar with reference to the intermediates in the pathway, but if differed in that S. pullorum, grown on sulfate, accumulated the intermediate compound sulfite, whereas, S. typhimurium wild type organisms did not. Also differing from S. pullorum in this respect is E. coli (62, 63).

Because of our interest in the cysteine biosynthetic pathway, information about the number and

location of the genes coding for the enzymes of the pathway was needed.

The primary purpose of this study was to extend the linkage map of S. pullorum to include the cysteine genes. Since Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) had developed a conjugation system in S. pullorum this technique was used for genetic mapping. During the course of this study a number of modifications were made with respect to the techniques for bacterial mating as described by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969).

One of the S. pullorum donor strains carrying the F-trp particle was further characterized with respect to its behavior as a chromosome donor.

A S. pullorum strain carrying an F-lac particle was isolated and characterized with respect to its donor ability.

All new mating systems must be rigorously examined to rule out recombination due to some phenomenon other than conjugation. A number of experiments were performed to rule out recombination due to spontaneous transformation or transduction. It is also important to demonstrate that recombinants are being produced and that growth of cells following mating procedures is not due to cross-feeding. A number of tests were performed to rule out cross-feeding.

LITERATURE REVIEW

Introduction

Since the discovery of bacterial conjugation by Lederberg and Tatum in 1946, many experiments have been reported and reviews written concerning the transfer of genetic information from one cell to another. The early history of bacterial conjugation as a means of genetic transfer was recounted by Lederberg and Tatum (70). More recently, reviews have been written by Falkow, Johnson and Baron (39), Novick (82) and Susman (102). These authors discuss the various aspects of the process of conjugation as well as the various types of conjugal fertility factors. Considering the fact that these rather extensive reviews are available, the material reviewed here will cover primarily the literature since 1968.

History

F^+ and Hfr donor types.--The original donor type discovered by Lederberg and Tatum was a cell in which the fertility factor was present autonomously in the cytoplasm. Subsequently, donors were isolated which produced a high frequency of recombination (Hfr) with F^- cells (46). The hypothesis that the Hfr donor type was produced by the

insertion of a specific factor into the chromosome was put forth by Jacob and Wollman (54). Campbell (16) proposed that this factor which was integrated was the F factor itself. The elements of the proposal included circular F and linear insertion of F into the chromosome. Broda (15) reported isolation of a number of Hfr cells from a single F^+ strain. Since these Hfr cells had different points of origin of transfer, Broda concluded that the F factor integrated into the chromosome at a limited number of sites on the bacterial chromosome. Genetic experiments performed by Curtiss (22) and physical studied by Friefelder (40) support the proposal.

F' donor type.--Still another relationship between the F particle and the chromosome was found. Jacob and Adelberg (56) reported that an isolate of an Hfr was found that transmitted F and Lac^+ at a high frequency and the chromosomal markers at a low frequency in the same sequence as the original Hfr. They proposed a particle composed of F and the genes for lactose utilization. These particles that associate chromosomal genes with the F particle are called F prime (F').

Chromosome Mobilization

Mobilization in F^+ donors.--One of the aspects of chromosomal transfer that remains unresolved is the mechanism by which the F factor, in F^+ strains, mobilizes

the chromosome. Jacob and Wollman (53) proposed that transfer in F^+ cultures was due to the presence of spontaneously occurring Hfr cells in the F^+ population. The report by Clowes and Moody (21) that chromosomal transfer by F^+ is greatly reduced from donor strains having a rec^- mutation would seem to support the theory of F integration (to produce Hfr cells). However, even though the donor carried a rec^- mutation, chromosome transfer was reported to occur in all cases at a fairly constant rate (however low) with any of the chromosome transfer factors. Curtiss and Renshaw (25) proposed the existence of two types of F^+ donors: Type I donors which give rise to stable Hfr donors, and type II donors which produce unstable donors. The difference between type I and type II donors was shown to be a property of the cell not produced by the F particle. Since type I and type II donors were equally capable of chromosome transfer, Curtiss and Renshaw suggested that the majority of recombinants in an $F^+ \times F^-$ mating must be produced by a mechanism which does not require a stable F integration into the chromosome. Data gathered by Curtiss and Stallions (26) showed that 15-16% of the recombinants formed in $F^+ \times F^-$ matings were due to the presence of stable Hfr donors in the F^+ population, the rest of the recombinants being produced by unstable or lethal integrations of F into the chromosome, or by a mechanism of

transfer that does not require F integration into the chromosome. Recently some doubt has been cast on the reliability of data using rec$^-$ mutants to support the position that the chromosome is transferred by unintegrated F. DeVries and Maas (34) reported that F-lac could be integrated into the chromosome of a recombination-deficient strain of E. coli.

Mobilization in Hfr donors.--Questions are also being raised with regard to some aspects of the proposed mechanism of transfer in Hfr donors. The mechanism proposed by Jacob and Wollman (55) involved the insertion of a specific factor (F) into the chromosome to produce the Hfr linkage group. The characteristics of the Hfr donor are given as follows: transfer of the chromosome begins at a specific point called the origin of transfer and markers are transferred in a specific sequence. The recombinants produced by Hfr donors are generally F$^-$; however, they may contain the sex factor if the entire donor chromosome is transferred to the recipient cell. The model for linearization of the chromosome proposed by Jacob, Brenner and Cuzin (57) predicts that when F is integrated into the chromosome to produce mobilization, part of the sex factor will be transferred proximally, and the rest distally. Since the recombinants produced by Hfr donors are generally F$^-$, as determined by lack of

donor ability and lack of sensitivity to male-specific bacteriophage, at least these functions of F must be carried distally. This distal portion of F could be a major portion, as 8-9 of the 12 known cistrons of F are associated with pili production (111), and pili are essential to male-specific phage production and genetic transfer (84).

Experiments by Wollman and Jacob (113) indicated that some recombinants from Hfr x F^- matings which had received terminal but not proximal markers from the Hfr parent showed peculiar donor properties. The interpretation given was that part of the F factor was transmitted proximally, part at the terminal end of the chromosome and that both pieces are needed for normal Hfr activity. The peculiar donor properties of the recombinants were attributed to the lack of some component of F that is transferred proximally.

Another piece of evidence used to support the idea that some F factor DNA is present on the lead region of the chromosome transferred by an Hfr was the low recovery of very early markers (less than 2 minutes) transferred by conjugation (72, 42). It was suggested by Pittard and Walker (91) that it was possible that sex factor DNA, present at the leading end of the donor DNA, exerted an antipairing effect which resulted in fewer recombinants for early markers. Experiments were devised (108) which

tested this theory by using as a recipient a cell with F DNA present. An Hfr in F^- phenocopy was mated with an isogenic Hfr and an early marker was selected. Since F DNA would be present in the recipient, there should be no reduction in recombination frequency for early markers. The low recovery of recombinants obtained in this experiment was interpreted to mean that the factor producing the low recombination frequency was not the presence of F DNA on the lead region of the transferred chromosome.

Recently (1972) these same authors (109) reported results of experiments designed to confirm the presence of sex factor DNA in the lead region of the chromosome transferred by Hfr donors. Their experiments provided no evidence for the proximal transfer of sex factor DNA by the Hfr used. In addition, they were unable to repeat the findings of Wollman and Jacob (113). Walker and Pittard state that the results of their experiments do not rule out the possibility that part of F is always transferred proximally but only integrated at very low frequency. The experiments do, however, contradict an experiment (Wollman and Jacob) which, in the past, has been frequently taken as evidence for the proximal transfer of part of F.

Physical studies reported by Glatzer and Curtiss (Abst. of the Annual Meeting ASM, p. 31, 1972) give evidence for the presence of F at the lead region of

conjugally transferred Hfr DNA. It appears that since genetic studies do not confirm the presence of F DNA at the lead region of the donor chromosome, that the F DNA transferred early does not code for any functions critical to the transfer of DNA.

Mobilization in F' donors.--It was noted by Jacob and Adelberg (56) that some cells in an Hfr population transferred a chromosomal marker adjacent to the F integration site at a very high frequency with the recombinant also receiving the sex factor. The same donor could also transfer the rest of the chromosomal markers at a low frequency and with the origin of transfer and orientation characteristic of the original Hfr donor. In these cells the F particle appeared to be autonomous as it was in F^+ donors, but it differed in that it appeared to have some chromosomal DNA associated with it. When these autonomous factors were introduced into a wild type F^- cell, a region of DNA homology would exist between the autonomous particle and the bacterial chromosome and this homology formed the basis for a model for chromosome mobilization by these particles called F prime (F') particles.

Scaife and Gross (99) proposed that mobilization of the chromosome produced by F' factors was due to the synapsis of the chromosomal DNA region of the F' with the homologous region of the chromosome to produce integration

and an Hfr type donor. Transfer by F' donors was reviewed by Scaife (100).

The F' particle is of particular importance to the Salmonella pullorum conjugation system. The development of the S. pullorum donor strains by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) was attained by introduction into S. pullorum of F' particles from strains of S. typhimurium. The introduction of F^+ particles into S. pullorum failed to produce chromosomal mobilization. If this lack of mobilization was due to the inability of the F^+ particle to produce linearization of the donor chromosome, it was reasoned that the introduction of an F' with chromosomal DNA might produce the homology necessary for F integration fulfilling the requirements of the model for transfer proposed by Scaife and Gross. S. pullorum donor cells were isolated following introduction of several F' particles carrying Salmonella genes.

Curing F' Factors

A characteristic of F' particles and other non-chromosomal DNA (plasmids) is that cells which no longer carry these particles can be isolated after treatment of the population with certain chemicals called curing agents. Among these agents are acridine dyes, ethidium bromide (EtBr), sodium dodecyl sulfate (SDS), rifampicin, and nitrosoguanidine (NTG) (9, 51, 93, 94, 95, 103, 104).

There are two modes proposed for the curing of plasmids. One involves the curing agent interfering with replication of the plasmid while the second involves the selection of F^- cells by the curing agent. The effect of ethidium bromide is probably by way of interference with DNA replication (94) since EtBr is known to bind to DNA (110) and since EtBr can cure a number of different types of plasmids such as F factors and R factors (12). Reports on curing experiments using sodium dodecyl sulfate (95, 104) seem to indicate that SDS acts as a selective agent for non-pilated cells. Cultures of bacteria actively synthesizing F pili are more sensitive to SDS than repressed cultures or F^- cultures. Some of the non-pilated cells selected have lost the plasmid associated with pili production. Tomoeda et al. (104) proposed that SDS may affect the cell membrane binding site for F or R replication and thus produce its curing effect. If SDS acts to select non-pilated cells, it might be proposed that it should also have a curing effect on Hfr cells. Inuzuka et al. (51) reported on the treatment of E. coli Hfr strains with SDS. Cells with various types of modified F factors were isolated as well as F^- cells. These authors concluded that SDS had some direct action on the F factor (producing modifications of F) as well as selecting for spontaneously produced F^- cells.

F Pili and Conjugation

The structure and function of F pili has been reviewed by Valentine et al. (105), and continues to be a topic for research and discussion. Brinton et al. (13) reported that mechanical removal of pili reduced the number of recombinants produced in matings. They also showed that both pili production and chromosomal transfer were reduced by growth in poor media. These findings linked the presence of F pili with chromosome transfer. The F pilus as revealed by the electron microscope is a thread-like extension from the cell surface with an average Diameter of 9.5 nm with a length of up to 20 μm (64). The pili seem to have a central dense line indicating an axial hole. F type can be distinguished from I type pili (produced by bacteria containing R factors) by their greater length and diameter and by the presence of end knobs (29). If pili are removed from the cell by mechanical means such as blending, they reappear very rapidly. Novotny et al. (83) reported that after removal of F pili by blending, the number of F pili per cell reached a constant level equal to 71% of the control culture in 4 to 6 minutes.

Two models for the function of F pili in conjugation have been proposed: the pilus conduction model of Brinton and the pilus retraction model of Curtiss.

In the pilus conduction model, Brinton (14) proposed the following steps: (1) collision of mating cells, (2) attachment of cells strong enough to resist disruption by dilution or pipetting, (3) transfer signal (the transfer signal is described as an event which occurs at the end of the F pilus which signals to the donor that chromosome transfer can begin), (4) transfer of DNA which occurs within the axial hole of the F pilus, (5) separation of mating pairs (there is a constant probability per unit time that the mating pair will spontaneously break and interrupt chromosomal transfer, (6) replication, recombination and beginning of gene function.

The pilus retraction model expressed by the Curtiss (24) proposes that the pili of the donor contact the recipient and then retract, pulling the cells into close contact at which point, a conjugation bridge is formed. These bridges were described by Anderson et al. (3) as cellular bridges of a diameter on the order of 100-300 nm. The pilus retraction model was favored by Marvin and Hohn (80) on the basis that phage adsorption to the pili or $F^+ \times F^-$ cell contact triggers a retraction of the pilus. Such a retraction would produce the close cell to cell relationship required for bridge formation.

Ou and Anderson (98) reported the results of experiments in which they observed and manipulated the mating pairs under the light microscope. Initial contact

between mating pairs of cells appeared to occur by means of one or more threads that are too thin to be visible in the light microscope (probably F pili). Mating pairs that were observed to be separated from each other during the mating period produced recombinants with donor markers. In addition, single Hfr cells were found to be able to conjugate simultaneously with two F^- bacteria even though they were separated for the entire mating period. Some mating pairs did achieve close cell to cell contact and these matings were about twice as fertile as those between separated pairs. These observations seemed to indicate that F pili are involved in the transfer of genetic information between Hfr and F^- bacteria. It also appeared as though two Hfr replication sites can be active in one Hfr cell. It would appear that transfer of genetic information can occur either through the F pili or through an opening (conjugation bridge) produced during close cell to cell contact.

Cells Containing Several Fertility Factors

The discovery that donor cells, when cultured to stationary phase, lost their donor ability and acted as recipient cells, paved the way for experiments introducing a second fertility factor into the bacterial cell.

Scaife and Gross (98) were able to show that following the introduction of an F-*lac* into an Hfr cell,

the multiplication of the F-lac was inhibited in the Hfr cell. In another similar experiment (77), only non-Hfr recombinants contained F-lac, whereas, F-lac was excluded from Hfr recombinants. On the other hand, it was also reported that an episome (F-lac) was capable of autonomous replication in an Hfr (27, 37).

The matings of two Hfr cells (19) produced cells that appeared to be "double males" having two different points of origin for mobilization when mated with F^- cells. Mass (78) also reported the isolation of a double male. When F-lac was introduced into an Hfr in F^- phenocopy, most of the recombinants were lac^- (F was excluded). However, one lac^+ recombinant was isolated in which it appeared that both sex factors were integrated into the chromosome. When an experiment was performed in which F-lac and F-gal were introduced into a single cell (30), only very seldom was a strain isolated which carried both episomes. These experiments seemed to indicate that two autonomous F factors could not replicate in the same cell but if integrated in the chromosome, two F factors may occupy the same cell.

Isolation of a strain carrying two sex factors not both integrated into the chromosome was again reported (28). One cell was reported to contain two F' factors in the autonomous state. Another was reported to contain an autonomous sex factor in an Hfr. It is possible that the

cell which appeared to maintain both F' factors actually harbored a fused particle made up to two F' particles. Fused F' particles which replicate as a single entity have been reported by Press et al. (92) and Willetts and Bastarrachea (112).

The explanation given by Dubnau and Maas (36) for the incompatibility between a resident, integrated F factor and a sex-duced, free F factor was that an inhibition of replication of the superinfecting particle was not due to restriction, but rather to a process similar to phage superinfection immunity. Another explanation for F factor incompatibility could be that the F replicator site proposed by Jacob et al. (57) is singular. If, in order to be replicated, the sex factor must occupy this site, then the replication of two sex factors would be precluded. Two integrated sex factors would escape the incompatibility barrier. Bastarrachea and Clark (8) reported the isolation and characterization of a triple male strain.

Maas and Goldschmidt (79) reported the isolation of a mutant of E. coli that permitted the replication of two F factors, one integrated and one autonomous. These authors contend that an F-lac in an Hfr may alternate between autonomous and integrated forms, but presumably it cannot replicate in the autonomous form. The mutant isolated was altered in its integrated F particle to

remove the incompatibility barrier. The mutant, however, would not maintain two unintegrated sex factors.

Compatibility between two F' factors in a mutant strain of E. coli was reported by Palchoudhury and Iyer (88). A cell containing a chromosomal mutation which caused an abrupt temperature-dependent arrest of DNA synthesis was isolated. This cell also had alterations in several other properties, all of which are membrane-associated. At the permissive temperature, this mutation conferred on cells harboring one autonomous F' factor an increased ability to allow the entry and stable maintenance of a second F' factor. These findings imply that the incompatibility between F factors is a membrane-associated phenomenon.

Integration of a second F factor into the chromosome does not insure its stable existence in the cell (61). From matings between two Hfr cells, over 400 strains were isolated that initially showed double male characteristics, yet all rapidly reverted to the Hfr state. The F factor which was lost was always that inherited from the Hfr donor parent. No stable derivatives were isolated from these matings. If F serves as the site for initiation of chromosomal replication, difficulties in chromosome replication might be the reason why the two integrated sex factors were not tolerated.

DNA Transfer During Conjugation

Curtiss (24) reviewed experiments performed to determine the functions of both donor and recipient cells during conjugation. Several questions remained to be answered. Is the transferred DNA single or double stranded? Is the replication of DNA associated with transfer, performed by the donor or the recipient bacterium? Does the donor push or the recipient pull the DNA during conjugational transfer?

The results of experiments by Vapnek and Rupp (106) answer some of the questions. These authors report confirmation of the report by Gross and Caro (44) which states that only one strand of the sex-factor DNA is transferred from donor to recipient during conjugation. This strand was shown to be transferred with a leading 5' end (50). The strand transferred was identified by Vapnek and Rupp (106) as the denser strand in CsCl-poly (U,G) centrifugation. A complement to this strand is synthesized in the recipient and a covalently closed sex-factor DNA molecule is formed. The sex factor strand not transferred to the recipient remains in the donor where it acquires a complement during mating and forms a covalently closed double-stranded circle. These results show that DNA synthesis associated with mating occurs in both donor and recipient cells. The

transfer signal and the precise functions of donor and recipient cells during transfer are yet to be discovered.

Chromosomal Transfer in Other Strains

Since the discovery of conjugation in *E. coli* by Lederberg and Tatum, the fertility factor has been introduced into a number of different bacteria in an attempt to produce a conjugation system for the purpose of genetic analysis.

The F factor was transferred from *E. coli* into several strains of *Salmonella* including *S. typhimurium* (5, 115), and *S. abony* (75). The behavior of the F factor in these strains is similar to its behavior in *E. coli*. The F factor in the form of F' particles was transferred from *S. typhimurium* to *S. pullorum* to produce a conjugation system in this strain (Godfrey, Ph.D. dissertation, Michigan State University, East Lansing, 1969).

Pasteurella pseudotuberculosis accepted the F-*lac* episome from *E. coli* and acted as a gene donor in crosses with several different auxotrophs of *P. pseudotuberculosis* (65). When F' plasmids were transferred from *E. coli* or *P. pseudotuberculosis* to *P. pestis*, the cell was able to transfer the F' to other cells but was unable to transfer chromosomal markers. However, a strain of *P. pestis*

carrying the F'Cm plasmid was able to donate chromosomal markers (66).

A mating system has also been described in Pseudomonas aeruginosa (47). Tests were performed to show that the marker transfer observed was due to conjugal transfer. The donor ability in P. aeruginosa was shown to be associated with the presence in the donor cell of a factor called FP (48). The FP factor differed from the E. coli F in that it was not curable with acridine orange treatment. The conjugation system in P. aeruginosa seems to function irregularly (71) which makes it difficult to use for genetic studies. Chromosome mobilization in another strain of Pseudomonas (Pseudomonas putida) was reported by Chakrabarty and Gunsalus (18). In this strain mobilization of the chromosome was reported to be associated with the presence in the cell of a defective phage particle.

Intergeneric Bacterial Matings

Intrageneric bacterial matings which first may have been done in an attempt to produce fertile donors in a number of bacterial strains are now being performed for a variety of other reasons.

Transfer of genetic material from E. coli into Proteus mirabilis has been very useful in determining the physical characteristics of F^+, F' and other DNA (6).

Transfer of DNA from one strain to another may also be used to create partial diploid strains of bacteria. The hybrid cell has been used to study the stability and function of DNA in a foreign host (6, 58, 59, 60, 67). Hybrid cells are being used to allow phage to infect a foreign host (because the hybrid has a new phage receptor site) so that the behavior of the phage DNA in the hybrid can be examined (7). Production of new phage receptor sites by hybridization also allows for transduction in strains in which no transducing phage was available. Intergeneric matings have also been used to locate various cistrons on the bacterial chromosome (85).

The fertility of intergeneric crosses is generally low (5). It is suggested by Mojica-a and Middleton (81) that there are at least three reasons for this low fertility: differences in cell surface, effects of female restriction on male DNA, and differences in the base sequences of DNA. The effects of these three factors are being studied at present because hybrids can now be produced at higher rates by mutants that have escaped the influence of these factors.

MATERIALS AND METHODS

Chemicals

N'-methyl-N'-nitro-N-nitrosoguanidine (NTG) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Ethidium bromide (EtBr), L-crysteinesulfinic acid (CSA), deoxyribonuclease 1 (pancrease) B grade (DNase) California Corporation for Biochemical Research.

Media

For routine cultivation of the bacteria, L broth and L agar were used. This medium contained 10 g tryptone (Difco), 5 g yeast extract (Difco) and 10 g NaCl per liter of distilled water. L agar contained in addition 1.5% agar (Difco). The L soft agar used for phage enumeration had 0.75% agar (Difco). Amino acid auxotrophs were grown using E minimal medium (107) supplemented with L-amino acids to a final concentration of 20 ug/ml and glucose at 0.4%. E minimal agar contained 1.5% agar (Difco) and E minimal soft agar contained 0.75% agar. Sulfate-free minimal medium was made by substitution of equimolar quantities of $MgCl_2 \cdot 6H_2O$ for the $MgSO_4 \cdot 7H_2O$ in E medium. Sulfate-free minimal agar was made by adding washed special Noble agar (Difco) to the E broth. The Noble agar was washed by adding 1 liter of deionized distilled

water to a flask containing 18 grams of agar and swirling it. The agar was allowed to settle to the bottom of the flask and the water decanted. The agar was washed in this manner three times before adding it to the medium. Various sulfur sources were added to this medium as described under methods for determining cysteine mutants.

For testing sensitivity to ultra violet irridation, the A minimal medium described by Hartman et al. (45) was used.

Bacto SIM medium (Difco) was used for the detection of sulfide and indole production. For streptomycin counter-selection, E minimal medium was supplemented with dihydrostreptomycin sulfate at a final concentration of 1000 µg/ml.

The stock solution of glucose (Pfanstiehl) used in these experiments was made up at 40% w/v with distilled water and sterilized by autoclaving at 15 lbs. pressure for 15 minutes.

Slant agar medium used for cell storage contained nutrient broth (Difco) 15 g, NaCl 5g, and agar (Difco) 25 g per liter of medium.

The medium used for penicillin selection called ESE was a modification of that described by Gorini and Kaufman (43). Flask A (250 ml) contained 50 ml of 1x E min salts. Flask B contained 20 g sucrose and 484 mg tris (hydroxymethyl) amino methane (TRIS) and distilled

water to a final volume of 50 ml at a pH of 7.2. After autoclaving, the contents of flask B was added to flask A and sterile glucose and $MgSO_4$ was added to a final concentration of 0.5% and 0.01 M respectively.

Galactose fermentation was determined on Levine EMB without lactose (BBL) supplemented with 8 g casamino acids (Difco) per liter. Filter-sterilized galactose was added to a final concentration of 0.8%. Lactose fermentation was determined on Levine EMB (Difco) supplemented with 8 g of casamino acids per liter. Occasionally the lactose medium was supplemented with 8 g tryptone instead of the casamino acids.

Enrichment medium was used to select for amino acid auxotrophs after mutagenic treatment. This medium consisted of E minimal medium supplemented with all L-amino acids at 20 µg/ml final concentration except for the amino acids required by the auxotroph under selection. In addition the medium contains calcium pantothenate, thiamine hydrochloride, guanine, adenine, cytosine, and uracil at a final concentration of 2 µg/ml, 100 mg of nutrient broth powder (Difco) and 15 g agar (Difco) per liter.

Bacteria

S. pullorum, S. typhimurium, and E. coli strains were used in this study and are described in Tables 1 and 2. The parental strain for all S. pullorum auxotrophs

TABLE 1.--Recipient strains of *Salmonella pullorum*.

Strain	Sex factor	Genotype	Source or Reference
MS35	F^-	*cys*-1 *cys*-2 *leu*-1	(62)
MS6	F^-	*cys*-1 *leu*-1	MS35 (62)
MS18	F^-	*leu*-1	MS6 (62)
MS350	F^-	*str*A1	MS18[a]
MS355	F^-	*str*A1 *trp*-2	MS350[a]
MS374	F^-	*str*A1 *pro*-1 *thr*-1 *ilv*A1 *gal*-1	MS350[a]
MS375	F^-	*str*A1 *pro*-1 *thr*-1 *ilv*A1 *gal*-1 lysogenic P22	MS374
MS81	F^-	*leu*-1 *cys*E1	MS18
MS90	F^-	*leu*-1 *cys*E1 *ilv*-3 *str*A2	MS81
MS91	F^-	*leu*-1 *cys*E1 *ilv*-3 *str*A2 *thr*-2	MS90
MS100	F^-	*leu*-1 *cys*B1	MS18
MS103	F^-	*leu*-1 *cys*B1 *trp*-3	MS100
MS104	F^-	*leu*-1 *cys*B1 *trp*-3 *his*-3	MS103
MS105	F^-	*leu*-1 *cys*B1 *trp*-3 *his*-3 *gal*-3 *str*A3	MS104

[a]Otis Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969).

TABLE 2.--Donor strains of *Escherichia coli*, *Salmonella typhimurium* and *Salmonella pullorum*.

Cell Type	Strain	Sex factor	Genotype
E. coli	AB785	F'	*met* / F-*lac*
S. typhimurium	SU694	F'	*trpA52 cysB12 pryF146 ilv-178* / FT71(*trp*$^+$)
S. pullorum	MS807	F'	*cys-1 cys-2 leu-1 trp-2* / FT71(*trp*$^+$)
	MS810	F'	*cys-1 cys-2 leu-1 his-4 trp-2* / FT71(*trp*$^+$)
	MS811	F'	*cys-1 leu-1 his-4 trp-2* / FT71(*trp*$^+$)
	MS812	F'	*leu-1 his-4 trp-2* / FT71(*trp*$^+$)
	MS920[a]		*leu-1 his-4 trp-2* / FT71(*trp*$^+$) F-*lac*
	MS921[a]		*leu-1 his-4 trp-2* / FT71(*trp*$^+$) F-*lac*
	MS814	F'	*leu-1 his-4 thy-1 trp-2* / FT71(*trp*$^+$)

[a]MS920 and MS921 were independently isolated from a mating between *E. coli* AB785 (F-*lac*) and MS900, the MS2 resistant cell isolated following treatment of MS810 with ethidium bromide.

produced was strain MS35, a naturally occurring double cysteine auxotroph (62).

Genotypes for all bacterial strains were designated in accord with the recommendations of Demerec et al. (33). Genotype was indicated by a three letter italicized symbol followed by a letter designating locus and a number indicating isolation number. Phenotype was indicated by the same three letter symbol with a capital letter and no italics. Phenotypes of the bacteria used are listed in Table 3.

Bacteriophage

The phage used in this study were the following: P22 amber mutant 11c1 from M. Levine, Dept. of Human Genetics, University of Michigan, Ann Arbor, Michigan; Felix anti-0, Ffm, P22.c2, and P221.c2 from B.A.D. Stocker, Dept. of Medical Microbiology, Standard University School of Medicine, Stanford, California; P35, a S. pullorum phage selected by zygotic induction from O. Godfrey, Eli Lilly Research Laboratories, Indianapolis, Indiana.

Mutagenesis

Cells were incubated at 37°C with aeration overnight in L broth and harvested by centrifugation, washed once with TM buffer (2) and resuspended in TM buffer pH 6.2 at a concentration of 1-5 x 10^7 cells/ml. NTG

TABLE 3.--List of genetic markers of *Salmonella pullorum*.[a]

Gene Symbols	Phenotypic Trait Affected	Reference
cys-1 cysteine	Sulfate permease	(62)
cys-2 cysteine	Sulfite reductase	(62)
*cysB*1 cysteine	Requirement for cysteine or sulfite, slow growth on CSA	(35)
*cysE*1 cysteine	Serine transacetylase	b
gal-1 galactose	Unable to ferment galactose	c
gal-3 galactose	Unable to ferment galactose	c
his-3 histidine	Requirement for histidine	c
his-4 histidine	Requirement for histidine	c
*ilvA*1 isoleucine-valine	Requirement for isoleucine or -keto-butyrate	
ilv-3 isoleucine-valine	Requirement for isoleucine	
leu-1 leucine	Requirement for leucine	(62)
pro-1 proline	Requirement for proline	c
*strA*1 streptomycin	High level resistance to streptomycin	c
*strA*2 streptomycin	High level resistance	
*strA*3 streptomycin	High level resistance	
thr-1 threonine	Requirement for threonine	c
thr-2 threonine	Requirement for threonine	
thy-1 thymine	Requirement for thymine	
trp-2 tryptophan	Requirement for tryptophan	c
trp-3 tryptophan	Requirement for tryptophan	

[a]Different acquisition numbers for the same mutant designation indicates that the mutants were isolated at different times; [b]W. D. Hoeksema, personal communication; [c]O. W. Godfrey, Ph.D. dissertation, M.S.U., East Lansing, 1969.

from a stock solution of 1 mg/ml in TM buffer was added to the cell suspension to a final concentration of 100 or 400 µg NTG/ml. The cells were incubated in this solution for 30 minutes at 37°C without aeration, removed from the NTG solution by centrifugation and washed two times with fresh TM buffer. The cells were either resuspended in L broth and incubated 4-5 hours to allow for segregation or placed in minimal medium supplemented to allow for growth of the desired auxotrophic mutant. The cells were then either plated on enrichment medium or the mutants were selected for by penicillin treatment.

Selection of Amino Acid Mutants

When the mutants were selected for by growth on limited enrichment medium (68), the mutagen-treated cells were diluted and plated to produce approximately 100 colonies per plate. After incubation the presumptive mutants (pinpoint colonies) were picked and patched on an L agar master plate. After incubation, the master plates were replica plated (69) onto media with and without the nutrient required by the presumptive mutants. Those colonies showing the desired growth patterns were selected as the mutants, and these cells were purified by streaking them for isolation three times.

Selection of Thymine-requiring Mutants

Thymine-requiring mutants were selected on solid media using the method described by Caster (17). The medium used was the same as described by Caster except that 0.5 g of trimethoprim was substituted for the aminopterin. S. pullorum cells were grown up in L broth, centrifuged, and resuspended in E minimal broth (1×10^8 cells/ml) and 0.1 ml of cells were spread on plates of thymine selection medium. The plates were incubated at 37°C for approximately 1 week before colonies were apparent. The mutants were identified by replica plating onto medium with and without thymine. The basic medium used was E minimal medium supplemented with 5 g casamino acids (Difco), 0.1 g tryptophan per liter and 0.4% glucose and 1.5% agar. Thymine was added to this medium at 40 µg/ml final concentration.

Penicillin Selection

The procedure of penicillin selection used was essentially that described by Gorini and Kaufman (43). Cells that had been treated with a mutagen were allowed to grow up in minimal medium supplemented to allow for the growth of the desired mutant. After incubation at 37°C with aeration to a density of approximately 1×10^9 cells/ml, the cells were centrifuged and washed with unsupplemented minimal broth. The pellet was resuspended

in E minimal medium. About 0.1 ml of the resuspended pellet was inoculated into E minimal medium (broth + 0.5% glucose). To this cell mixture was added nutrients to make the medium complete as possible with reference to nutrients required by auxotrophs other than the one desired. The total volume of the cell nutrient was 5 ml, less that volume needed for the addition of penicillin. The cell nutrient mixture was added to 5 ml of ESE medium and the mixture incubated at 37°C with aeration until the population doubled (3 hrs). Penicillin was then added to a final concentration of 2,000 units/ml and the mixture incubated at 37°C without aeration. The incubation period was that time necessary for 50% of the cells to form spheroplasts (3.5-4 hrs). The cells were chilled, centrifuged, and resuspended in 10 ml of E minimal medium supplemented for the growth of the desired mutant. After incubation, the desired mutant was selected on limited enrichment media, or the cells were recycled through the penicillin selection procedure.

Selection of Cysteine Prototrophs of *S. pullorum*

S. pullorum wild type MS35 and the donor strain MS810 were reverted to cysteine prototrophy using the method of Kline and Schoenhard (62). The wild type *S. pullorum* cysteine mutants were reverted to sulfite

utilization by growing them at 37°C on a plate of E minimal medium without sulfate, supplemented with CSA on which several crystals of NTG had been placed. The recipient revertant was designated MS6 and the donor revertant, MS811. The S. pullorum prototroph was produced by growing the first revertant on E minimal medium at 37°C with crystals of NTG on the plate. The recipient prototroph was designated MS18 and the donor prototroph MS812.

Methods for Characterizing Cysteine Mutants

Some of the cysteine mutants produced were characterized using the auxanographic method devised by Beijerink as described by Lederberg (68). The bacteria to be tested were grown 24 hours in E medium supplemented with cysteine. The cells were harvested by centrifugation and washed 2x in an equal volume of 1x E salt solution. A tube of E minimal soft agar was inoculated with 0.3 ml of washed cells and overlaid on a sulfur-free E minimal medium plate. A sterile filter disc of 6 mm diameter was placed on the surface of the overlay and impregnated with 50 µg of a sulfur compound. Stock solutions were made up so that 0.1 ml fluid was placed on the filter discs. The compounds tested in this manner were sodium sulfite (Na_2SO_3), sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) and cysteine sulfinic acid (CSA). The plates were set up in duplicate and incubated at 25 and 37°C. Plates were scored after

3-4 days of growth. Sulfide utilization was tested by adding cells prepared as above to a screw-cap tube of sulfate-free E medium and adding sodium sulfide to a final concentration of 2×10^{-4} M. A control tube containing only sulfate-free E medium was also inoculated as occasionally the cells would grow with no added sulfur source. This growth is probably due to the presence of sulfur as a contaminant in the components of the E medium. The tubes of sulfide and control tubes were sealed by wrapping the tops of the tube with parafilm to prevent loss of sulfide as it is highly volatile. These tubes were scored after 1-2 days of incubation at 37°C without shaking.

The Cysteine Mutants

The mutant designated cys-1 will grow on E minimal medium supplemented with cysteine, sulfide and CSA but not on sulfate as sulfur source. The cys-2 mutant will grow on E minimal medium supplemented with sulfide, but when supplemented with CSA it will grow at 25°C but not at 37°C. The following mutant types were given letter designations because they appeared to be similar to S. typhimurium mutants as described by Dreyfuss and Monty (35). The cysB1 mutant will grow on E minimal medium supplemented with cysteine or sulfide, and grows slowly on CSA. This cysteine mutation is also co-transducible with trp. The cysE1 mutant grows on E

minimal medium supplemented with cysteine, but not when supplemented with any of the intermediates of the cysteine biosynthetic pathway. This mutant showed less than 10% wild type serine transacetylase activity (W. Hoeksema, personal communication).

Test for the Presence of F

Bacteria were tested for the presence of the F factor by determining their sensitivity to the donor specific RNA bacteriophage MS2 (101). Approximately 0.04 ml of an MS2 phage lysate containing about 1×10^{11} plaque forming units (PFU) per ml was streaked down the center of an L agar plate using a 0.2 ml pipette and the streak was allowed to dry. The cells to be tested were grown in L broth and subcultured into 3 ml of fresh L broth in a 13 x 100 mm test tube. These cells were incubated 3-4 hours at 37°C without aeration prior to the test. The cells were then streaked across the phage streak using an inoculating loop. Cells carrying F showed greatly reduced numbers where they were pulled across the MS2 streak: F^- cells showed no reduction in number. These plates were read in 5-6 hours, as longer incubation times resulted in a loss of the distinction in growth between the cells having F and those that do not carry F. The MS2 phage lysate used in this test was prepared by adding phage to a screw-cap tube of L broth containing

E. coli Hfr H cells at about 1 x 10^7 cells/ml. This mixture was allowed to incubate for 3-4 hours at which time 0.5 ml of chloroform was added to the tube and the tube was mixed on a vortex mixer. The lysate was stored over chloroform at 5°C.

Techniques for Bacterial Mating

Millipore mating.--Early in this study, bacterial matings were done as described by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969). Later, modifications of the procedure were made following those described by Curtiss et al. (23). Donor and recipient cells were grown overnight in L broth at 37°C with aeration. These cultures were started with isolated colonies of the cells which had been grown on L agar and then stored in the refrigerator. The donors and recipients were diluted 1:20 into fresh L broth in 18 x 150 mm tubes and the recipients were incubated for 3 hours at 37°C with aeration. Two ml portions of the diluted donor culture were placed in 13 x 100 tubes and incubated for 3 hours at 37°C without aeration. Five mls of the recipient cell culture (1 x 10^8 cells/ml) was added to the 2 mls of the donor cells (1 x 10^8 cells/ml). The suspension was gently mixed and drawn down onto a membrane filter (Millipore HA 0.45μ, 25 mm diameter) that had been pre-wet by drawing several mls of a sterile 0.85% saline

solution through the filter system. The filter and cells were removed from the apparatus immediately after all fluid was drawn down into the filter, and placed firmly on the surface of a pre-warmed L soft agar plate. The plate containing the membrane was incubated at 37°C for the desired mating time. After incubation, the mating pairs were disrupted (see section on disruption of mating pairs) and the cell suspension was appropriately diluted in E minimal broth. One tenth ml portions of the cell suspension were placed in tubes containing 3 mls of E soft agar which was melted and maintained at 45°C in a water bath. The contents of each tube was mixed and then poured onto the surface of E minimal medium agar plates properly supplemented to allow only for growth of the recombinant cells. Unmated donor and recipient cells were also plated as controls for back mutation. The plates were incubated 4 days and the number of recombinant cells recorded. When this technique was used for interrupted matings, 0 time was taken as that time when the filters were placed on the surface of the L soft agar plate.

Broth matings.--Donor and recipient cells were grown overnight in L broth at 37°C with aeration. The recipient cells were subcultured into 8 ml of fresh L broth in 18 x 150 mm tubes and incubated at 37°C with aeration. The size of this inoculum was usually about

0.5 ml. Donor cells were also subcultured into 8 ml of fresh L broth and then 1 ml portions were pipetted into 13 x 100 mm tubes and incubated at 37°C without aeration. The inoculum of donor cells used was about 0.2 ml as the donor cells grew to a higher cell concentration in an overnight culture. This inoculum produced about 2 x 10^8 cells upon a 3 hour incubation. After the incubation period the tube of donor cells was gently poured into the tube of recipients and this mixture was gently poured into a 250 ml Erlenmeyer flask and incubated at 37°C. Originally the matings were done without agitation, but it was noted that after 30 minutes of mating the cells began to settle out. Following this discovery, the flasks were agitated during the mating in a New Brunswick G-76 gyrotory shaker bath at a shaker setting of 2.5. After the appropriate mating period the mating pairs were disrupted and appropriate dilutions were made and the cells plated as in Millipore matings.

Cross-streak matings.--The procedure was essentially that described by Berg and Curtiss (10). The cells were prepared as for the other matings. Using a 0.2 ml pipette approximately 0.04 ml of recipient bacteria were streaked down the center of a plate of media appropriate for selecting recombinants. After the streak had dried, donor cells were streaked across the recipient streak using an inoculating loop. Several donors were tested

per plate. After these streaks had dried, the plates were incubated at 37°C and recombinants scored after 3-4 days. This method was useful only for the high frequency S. pullorum donors such as MS810. In other matings the recombination frequency was too low to be detected by this method.

Disruption of Matings Pairs

Millipore mated cells were disrupted by placing the filter with its cells into a 13 x 100 mm test tube containing 2 ml of E minimal broth and agitating the tube for 60 seconds using a vortex mixer. The liquid was then poured into an 18 mm fluted screw-cap test tube and agitated on the mixer for 2 minutes to complete disruption of the mating pairs. In broth matings the sample was placed directly into the fluted tube and agitated for 2 minutes to disrupt the mating pairs. Since some experiments seemed to indicate disrupting of the mating pairs was incomplete, the apparatus described by Low and Wood (74) was made for disruption of mating pairs. When this apparatus was used for disruption of cells that had been Millipore mated, the filter disc was placed in a 13 x 100 mm tube with 2 ml of E minimal broth. The vibrating apparatus was turned on for 15 seconds (experiments showed this to be adequate for disruption) and the cells were then diluted and plated as described previously. For disruption of broth matings, the broth

sample removed from the flask was placed directly into a 13 x 100 mm tube and this tube was vibrated in the apparatus for 15 seconds. Samples were removed and diluted and plated as described previously.

Selection for Unselected Markers

Recombinants were selected at random from the plates on which they were growing and purified by streaking for isolation on the same medium on which they were initially selected. After incubation (3-4 days), single colonies were spread in patches on plates of the same medium as previously used and incubated at 37°C for 2-3 days. These patch plates were used as master plates for replica plating the cells to test media for determination of unselected markers. The replica plates were incubated 3-4 days at 37°C and scored for growth.

Test for Stability of Recombinants

Recombinants were picked from unselected marker plates and transferred to L broth. After growth at 37°C with aeration to a maximum turbidity (approx 1×10^9 bacteria/ml) a loopful of cells was transferred to a tube of fresh media. This procedure was repeated three times. The cells were then streaked in patches on L agar and replica plated to determine their phenotype.

Test for Transfer of
cys-1 and cys-2
during Conjugation

These markers were mapped by testing for the expression in the recombinant of the mutant phenotype using unselected marker analysis. The plates on which the recombinants were selected and purified were supplemented with cysteine. The master plates were replica plated onto two plates each of E minimal medium and E minimal medium supplemented with CSA. One plate of each type of medium was incubated 37°C and the other at 25°C. Recombinants were scored as follows:

		growth on CSA		growth on SO_4^{-2}	
		25°	37°	25°	37°
cys-1^+	cys-2^+	+	+	+	+
cys-1^-	cys-2^+	+	+	-	-
cys-1^+	cys-2^-	+	-	+	-
cys-1^-	cys-2^-	+	-	-	-

The plates incubated at 37° were read in 4 days, the plates at 25° in 6-7 days.

Tests for Crossfeeding

When matings were done, media for the selection of recombinants was also seeded in the following manner. (1) Plates were overlaid with E soft agar containing 0.1 ml donor cells or 0.1 ml recipient cells. (2) Plates were overlaid with E soft agar containing both 0.1 ml

donor and 0.1 ml recipient cells. (3) An E soft agar overlay was inoculated with either 0.1 ml donor or recipient cells and poured onto the plate. Then a membrane filter (Millipore membrane HA 0.45 μ, 47 mm diaeter) was placed on the center of the plate and after the overlay had solidified, 0.1 ml of the opposite mating type cell was added to a second E soft agar tube. This suspension was placed on the top of the membrane filter using a sterile pipette. These plates were incubated at 37°C and observed for growth along with the other plates from the mating experiments. Another test involved placing parallel streaks of donor and recipient cells on media designed to select recombinants. During the selection for unselected marker experiment, another test was performed. One of the plates on which the recombinants were placed was supplemented so as to allow for the growth of the donor cell. This would allow detection of any colony that grew because it was contaminated with donor cells. In addition, a plate of unsupplemented media was used to detect if any recombinant had received all the donor markers. This plate was included in the test plates, as any cell that would grow on unsupplemented media would also grow on the media designed to check for donor contamination when auxotrophy was used for counterselection. If this plate were not included, all prototrophic recombinants would appear to be donor contaminated colonies.

Negative Control for Spontaneous Transformation

Donor and recipient cells were grown up as for a broth mating. A stock solution of DNase was made up at 2 mg/ml. A quantity of this stock solution was added to the tube containing the donor cells prior to mixing with the recipient cells. The final concentration of DNase in the mating mixture was 20 µg/ml. Cells were mated, disrupted, and plated as described previously.

Transduction

Transduction studies were made using a phage P35 isolated from S. pullorum by zygotic induction. The phage was purified by three separate plaque isolations on the donor strain. A plaque was fished to a screw-cap tube of L broth containing approximately 1×10^7 donor cells. After three hours of incubation with aeration at 37°C, 0.5 ml of fresh cells was added to the tube and the tube was reincubated. After 3 hours, 0.5 ml of chloroform was added to the culture tube and the contents were agitated for 30 seconds on the vortex mixer. This phage lysate was titered on the donor strain and stored over chloroform at 5°C without further treatment. For transduction, recipient bacteria were grown up in L broth to a titer of 1×10^9 cells/ml. One ml of recipient cells was placed in a 13 x 100 mm tube in a temperature bloc set at 37°C and transducing phage was added at a multiplicity of

infection (m.o.i.) of one. This phage cell mixture was incubated at 37°C in still culture for 15 minutes, after which 0.1 ml portions were placed in 3 ml of E soft and plated as in the mating experiments. The recipient was tested for reversion and the phage lysate for contamination with donor cells. The transduction frequency is defined as the number of transductants per phage in the transduction mixture.

Transduction Using Lysates Prepared from Mating Mixtures

The donor MS810 and recipient MS374 were grown up as they were for mating experiments. The cells were mixed and incubated at 37°C as a broth mating in a 250 ml Erlenmeyer flask. After incubation, the contents of the flask were poured into an 18 x 140 mm tube. About 1 ml of chloroform was added and the mixture agitated on a vortex mixer for 60 seconds. The chloroform was allowed to settle out, and 0.7 ml of the mating mixture lysate was placed in each of two 13 x 100 mm tubes (A and B) resting in a temperature bloc at 37°C. To tube A was added 0.1 ml of DNase solution (5 mg in 0.25 ml 0.85% saline), to tube B, 0.1 ml of 0.85% saline. After 20 minutes incubation, chloroform was added to each tube. The tubes were agitated on a vortex mixer and the chloroform was allowed to settle out. The recipient, MS374, was grown as for transduction

and 0.5 ml was placed in each of two 13 x 100 mm test tubes in a temperature bloc at 37°C. To the first tube was added 0.5 ml of phage lysate from tube A and to the second, 0.5 ml of phage lysate from tube B. These mixtures were allowed to incubate for 30 minutes and 0.1 ml portions were plated on appropriate media in E soft agar overlays.

Test for the Presence of Suppressors

Several P22 phage containing amber mutations which prevented phage muturation, and a strain of S. typhimurium designated 192, capable of suppressing this mutation, were obtained from M. Levine, Dept. of Human Genetics, University of Michigan, Ann Arbor, Michigan. Since P22 will grow on S. pullorum as well as S. typhimurium this phage was used to determine if certain S. pullorum mutants contained suppressors. Phage 11c1 was grown up on S. typhimurium 192 to a titer of 3×10^{10} PFU/ml. The cells to be tested were grown overnight in L broth. The test was performed by adding 0.1 ml cells and 0.1 ml of phage (at various dilutions from 10^{-9} to 10^{-1}) to a 3 ml soft agar overlay (1). After 12-24 hours of incubation plaques were counted. The titer on S. typhimurium 192 was taken as indicative of a strain capable of suppression and the titer on S. typhimurium LT2 as the non-suppressible background level.

There generally was a million-fold difference in the number of plaques produced on these two strains of cells. Those S. pullorum strains that produced phage titers comparable to those produced on S. typhimurium 192 were considered to contain a suppressor for the amber mutation.

Test for Lysogeny

The tester phage described by Gemski and Stocker (41) were obtained and used to determine whether or not certain strains of S. pullorum were lysogenic. In addition these phage were used to determine the surface characteristics of S. pullorum strains.

Phage Adsorption Test

This test was performed essentially as described by Adams (1). Cells to be tested were grown up to approximately 1 x 10^9 cells/ml in 10 ml of L broth. To this tube phage was added to a m.o.i. of 1 and the contents of the tube mixed on a vortex mixer. After the appropriate incubation time at 37°C, a 1 ml sample was removed from the tube and 0.5 ml of chloroform was added. The mixture was agitated on a vortex mixer for 60 seconds and the chloroform was allowed to settle out to the bottom of the tube. The number of free phage was titered by placing appropriate dilutions of this supernatant into 3 ml L soft agar along with 0.1 ml of susceptible cells

1 x 10^9 ml) and pouring this soft agar on a L agar base plate. S. typhimurium 192 known to have the adsorption site and S. typhimurium LT2 fer2 which lacks the P22 adsorption site were used as controls.

Plaque Resolution

The method of Pattee (89) was used to aid in the enumeration of plaques. Plates containing phage and cells in a soft agar overlay were flooded with 10 ml of L broth containing 0.1% 2,3,5,triphenyltetrazolium chloride and incubated at 37°C for 20-30 minutes until the cellular background had developed a red color. The excess tetrazolium solution was poured off and the plaques were counted.

Curing of F Using Ethidium Bromide

The curing method of Bouanchaud et al. (12) was used. The growth medium was prepared by adding 9 mg of ethidium bromide (EtBr) to a tube containing 9 ml of L broth. This solution gave a concentration of 2.5 x 10^{-2} M EtBr. Serial 1/10 dilutions were made in L broth to determine the highest concentration of EtBr that would allow good growth from a inoculum of 1 x 10^4 cells/ml. The appropriate concentration was found to be 2.5 x 10^{-3} M EtBr. After growth in this medium to approximately 1 x 10^9 cells/ml, cured cells were selected.

Determination of Phage Production by Zygotic Induction

Donor and recipient cells were grown as they were for mating experiments and 0.1 ml of each was added to an E minimal soft agar overlay, which was plated on media designated to select recombinants. When the donor was carrying a phage that the recipient did not carry, plaques were produced in the haze of background growth due to the 0.2 ml of L broth in the overlay. Zygotic induction was also determined by assaying the supernatant from a broth mating for plaque forming units. A third assay involved observation of lysis of recipient cells on a cross streak mating plate. At the point where the donor streak crossed the recipient cells, a zone of lysis developed if phage were produced by zygotic induction. The zone of lysis on the recipieint streak extended approximately 2 mm above and below the intersection of the donor and recipient streaks.

Test for Ultra Violet Sensitivity

The ultra violet sensitivity of bacterial cells was determined using the technique described by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969). Cells in the logarithmic phase of growth in L broth and at a concentration of 1×10^8 cells/ml were centrifuged and resuspended in an equal volume of

A minimal broth. A 3 ml sample was placed in the bottom half of a glass petri dish (100 mm diameter) at 48 cm from a 30-watt General Electric 630T8 germicidal lamp. During the time of exposure the top of the petri dish was removed and the cell suspension was agitated to promote uniform exposure. After exposure the cell suspension was immediately diluted in saline and plated on L agar. Post exposure manipulation of the cells was done in yellow light to prevent photoreactivation. The plates were wrapped in aluminum foil, incubated at 37°C and counted after 24-48 hours.

Electron Microscopy

The cells to be examined for the presence of F pili were grown overnight in L broth at 37°C with aeration. Approximately four hours before filtration, a 0.5 ml sample was subcultured into fresh L broth and incubated at 37°C without aeration. The culture was filtered through a Millipore membrane filter HA 0.45 μ of 25 mm diameter. The filter disk with the cells was placed in a 13 x 100 mm test tube containing 3 ml sterile deionized water. The tubes were gently shaken to place the cells in suspension.

Approximately 2 hours later, a suspension of MS2 phage was added at an m.o.i. of 100. After a 15 minute incubation to allow for phage adsorption, a drop of the sample was placed on a formvar grid. The sample was

stained with a 0.5% phosphotungstic acid solution at pH 7.5 for approximately 5 minutes.

The grids were examined visually in a Phillip's EM3000 electron microscope. Photographic records were made by exposing and developing Estar thick base plastic film (3 1/4 x 4 in.).

RESULTS AND DISCUSSION

Extension of the Genetic Map of S. pullorum to Include Four Cysteine Loci

In order to extend the genetic map of S. pullorum, particularly with respect to the cysteine genes, approximately 100 cysteine mutants of S. pullorum prototrophic recipient types were isolated following mutagenic treatment of several cultures with NTG. These mutants were classified into four groups by auxanographic tests (see Materials and Methods) and purified by three successive streaks for isolation. In order to establish the relationship between the cysteine genes and other markers, other amino acid mutations were added to the cells classified as cysteine mutants. These multiple auxotrophs were used as recipients in matings with the donors MS810, MS812 and MS814. These donors all carry the F-trp factor FT71 but differ with respect to their chromosomal markers.

The cysE1 locus.--The data collected from matings between S. pullorum donors and recipients carrying the cysE1 mutation are recorded in Table 4, and selection for unselected marker data on the recombinants of these matings are recorded in Table 5.

TABLE 4.--Number of $CysE^+$, Ilv^+ and Thr^+ recombinants per ml mating mixture in a three hour Millipore mating of *S. pullorum* strains.

Mating Strains	Marker Selected	Number of Recombinants per ml Mating Mixture
MS812 x MS90	Cys^+	7.6×10^4
	Ilv^+	2.0×10^5
MS810 x MS90	Cys^+	8.9×10^4
	Ilv^+	1.8×10^5
MS812 x MS91	Cys^+	6.4×10^2
	Ilv^+	1.4×10^4
	Thr^+	1.6×10^4

TABLE 5.--Linkage analysis of recombinants from a three hour broth mating.[a]

Unselected Marker	Selected Marker		
	Ilv^+ 206[b]	Thr^+ 276	Cys^+ 93
Ilv^+	--	37.0%	48.0%
Thr^+	73.0%	--	54.0%
Cys^+	4.5%	4.8%	--

[a]Data from a mating between MS812 and MS91.

[b]Number of recombinants tested.

The number of recombinants produced using the cysteine auxotrophic donor MS810 was approximately equal to the number produced when the cysteine prototrophic donor MS812 was used (Table 4). These results indicate that the cys mutations in MS810, cys-1 and cys-2 are not closely linked to the cysE1 region. If these cys markers were closely linked, the number of Cys^+ recombinants produced using the MS810 auxotrophic donor would be lower than the number produced using the MS812 prototrophic donor because of counterselection for recombinants receiving the donor cys mutations.

Gradient of transfer data from the MS812 and MS90 mating (Table 4) suggest that ilv-3 is closer to the origin of mobilization in donors carrying FT71 than is cysE1.

The addition of the thr-2 mutation to the recipient producing the strain designated MS91 allowed for the establishment of the order of entry for the FT71 donor as 0-thr-ilv-cysE based on number of recombinants produced (Table 4). This order of entry was also supported by the unselected marker data (Table 5).

The thr-2 mutation seemed to affect some process in the cell in addition to making it unable to synthesize its own threonine because when MS91 was the recipient, the recombination frequency for all markers was reduced when compared to the recipient MS90. In addition, the

recombinants produced when MS91 was the recipient seemed to grow more slowly and exhibit an instability. When 400 presumptive Cys^+ recombinants were picked for unselected marker analysis, only 187 remained when the patch plates had incubated. Of 392 Thr^+ recombinants picked, 300 grew on the patch plates and of 384 Ilv^+ recombinants, 221 showed growth on the patch plates. The loss of recombinants in these matings was much higher than is usually found for S. pullorum recombinants.

Some of the recombinants from an MS812 x MS91 mating were tested for the presence of the F factor by use of the MS2 phage sensitivity test. It appeared that more prototrophic than non-prototrophic recombinants were sensitive to MS2 phage. Although these data were from a prolonged mating of three hours, terminal markers were not selected. The relationship between prototrophy and MS2 sensitivity indicates transfer of the entire chromosome, including F, by an Hfr type donor.

The close linkage of Ilv^+ and Thr^+ in the recombinants and their instability as well as MS2 sensitivity suggest the presumed recombinants are merodiploids. The production of merodiploids indicates that the recombination function in MS91 had been impaired (73). Since recombination-less strains of bacteria lack the enzymes necessary for ultra violet (UV) repair, they exhibit an increased sensitivity to UV damage (20, 49). The UV

sensitivity of MS91 was tested and found to be similar to that of wild type S. pullorum MS35, which is able to produce recombinants (data not reported). It may be that in these cells, the recombination function has been altered without greatly affecting the UV sensitivity of the cell.

A problem which must always be considered in conjugation studies is that of cross-feeding between donor and recipient bacteria. When donors are counter-selected by a single auxotrophic requirement, the possibility for cross-feeding is present. Counter-selection in the E. coli and S. typhimurium conjugation systems uses male-specific lytic phage in addition to auxotrophic markers. At present, we have not developed a phage system that can be used in S. pullorum, so we are dependent upon auxotrophy or antibiotic sensitivity for counter-selection.

Since cross-feeding could be responsible for the apparent instability of recombinants in matings with the recipient MS91, a thy mutation was added to the donor strain MS812 to produce the MS814 strain. The thy mutation was chosen because on the S. typhimurium genetic map (96). This marker maps distal to the markers that were being transferred to the MS91 recipient by the F-trp donor, and there is reason to believe that the S. pullorum genetic map is quite comparable to that of S. typhimurium. The use of MS814 in mating experiments in which it was

counter-selected by the omission of thymine and histidine did not alter the frequency of recombination from that obtained with MS812. The thy mutation was unstable and the use of MS814 was discontinued.

A question that remained to be answered was the following: had the cysE locus really been mapped or had the locus of a suppressor been mapped? The question arose because of the use of the MS812 strain. The MS812 strain was produced by a two step NTG induced reversion of a double cysteine auxotroph MS810. It is possible that the reversion to cysteine prototrophy results from the suppression of one or both of these mutations. (A more thorough examination of this question was made later. See pages 59 and 62.) Since matings using the MS810 (non-revertant) donor produced approximately the same recombination frequency as matings using MS812, it seems reasonable to conclude that a suppressor is not interferring with the mapping of cysE.

The cysB1 region.--Two mutants carrying the cysB1 mutation, MS102 and MS105 (Table 1) were employed in the study of the cysB locus. Matings between these cells and MS812 were performed and Cys^+ and Trp^+ recombinants were selected. The data are recorded in Table 6. Analysis of these data indicate that $CysB^+$ recombinants are produced in matings with the F-trp donor at a lower

TABLE 6.--Recombination frequency for $CysB^+$ and Trp^+ in a three hour Millipore mating of S. pullorum.

Mating Strains	Marker Selected	Recombinants per Initial Donor Cell
MS812 x MS103	Cys^+	8.0×10^{-7}
	Trp^+	1.4×10^{-4}
MS812 x MS105	Cys^+	1.3×10^{-6}
Ms810 x MS103	Trp^+	4.4×10^{-4}

frequency than Trp^+ recombinants, and that the recombination frequency for these markers is lower than for other chromosomal markers (Table 11).

It was expected that $CysB^+$ and Trp^+ would be transferred at approximately the same frequency since they are co-transducible (Godfrey, Ph.D. dissertation, Michigan State University, East Lansing, 1969). The reduced recombination frequency for $CysB^+$ is indicative of its transfer as a very early marker or as a terminal marker on the chromosome. A reduced recombination frequency for markers transferred near the origin of transfer was reported by Low (72) and Glansdorff (42). The experiments reported show that in E. coli, early markers are transferred at about 10% of the frequency expected from transfer gradient information and that markers 4 to 5 minutes from the origin are not affected. The fact that the frequency of transfer for $CysB^+$ is much less

than 10% that of Pro^+, a marker transferred in about 40 minutes by the F-trp donors, indicates that $CysB^+$ is transferred by the F-trp donor as a terminal marker. The position of cysB on the genetic map would be adjacent to trp and in the case of the F-trp donor, apparently the F-DNA integrates between these two loci. The slight depression in the frequency of transfer Trp^+ when compared to Pro^+ may be a reflection of its proximity to the origin of transfer in the F-trp donor. The transfer of $CysB^+$ by the F-trp donor is an indication that the entire chromosome is transferred by this donor. The data on $CysB^+$ and Trp^+ transfer also can be taken to indicate that the chromosome in S. pullorum is circular.

The cys-1 and cys-2 loci.--A primary isolate of S. pullorum was found to be a double cysteine mutant by Kline and Schoenhard (62). They found S. pullorum MS35 to be a double auxotroph, lacking the ability both to transport sulfate into the cell (cys-1) and to reduce sulfite to sulfide (cys-2). In order to map these two genes, a recipient having these two mutations was mated with a prototrophic donor (a revertant from cysteine auxotrophy in our system since wild type S. pullorum MS35 is a double cysteine auxotroph). However, the possibility exists in such a system that the reversions to cysteine

prototrophy are not true reversions but reversion due to suppression.

Since the methods available could not be used to determine if the S. pullorum donor MS812 contained the amber suppressor (see page 62), cys-2 and cys-1 were mapped indirectly.

A mating was set up between a S. pullorum donor having the two cys mutations (cys-2 and cys-1) and the recipient MS374, a pro, ilv, thr mutant. From this mating, Pro^+, Ilv^+ and Thr^+ recombinants were selected and analyzed for the presence of cys-2 and cys-1 as unselected markers. (See Materials and Methods section for test for transfer of cys-2, cys-1.)

Since the trasnfer of cys-2 and cys-1 was analyzed by the unselected marker analysis (Table 7) exact placement of these markers on the genetic map was not possible, but it was possible to establish the position of these cys markers with respect to the selected markers Pro^+, Ilv^+, and Thr^+. The cys-2 region was located between ilv-1 and thr-1, and the cys-1 region was located further from the origin of transfer than thr-1, but not as far as cysB1.

TABLE 7.--Linkage analysis of recombinants from a three hour broth mating.[a]

Unselected Marker	Selected Marker		
	Pro$^+$ 1756[b]	Ilv$^+$ 1182	Thr$^+$ 1084
Gal$^+$	41%	35%	43%
Pro$^+$	-	41%	29%
Ilv$^+$	15%	-	48%
cys-2	7%	27%	35%
str	2.7%	22%	13%
Thr$^+$	1.5%	15%	-
cys-1	2.8%	8%	12%

[a]Data from a mating between MS810 and MS374.

[b]Number of recombinants tested.

Analysis of Salmonella pullorum Revertants for the Presence of Suppressors

Since wild-type S. pullorum MS35 is a double cysteine auxotroph (62), to map these cys loci, revertants to cysteine auxotrophy were produced by NTG mutagenesis. The cysteine prototrophs produced may be true revertants or revertants due to suppression. The presence of suppressors can interfere with the mapping of the cys loci so attempts were made to test the cysteine prototrophs for the presence of suppressors.

The method employed was infection of the cell in question with P22 phage having the amber mutation. This phage will produce a normal burst of phage when it infects a cell having an amber suppressor, but will produce only a small background number of plaques in a cell that does not have the suppressor. A P22 phage containing an amber mutation (phage 11c1) was obtained from M. Levine (Department of Human Genetics, University of Michigan, Ann Arbor, Michigan) and used to infect various S. pullorum strains. The data recorded in Table 8 show that, of the S. pullorum recipieits tested, wild-type MS35 and the revertant of cys-2 (MS6) did not contain an amber suppressor, whereas the prototrophic MS18 was able to suppress the P22 amber mutation. These data indicate that the reversion of the cys-1 mutation may be due to suppression and that the cys-1 mutation may be an amber mutation. Since the recipient MS374 used to map cys-1 and cys-2 is a derivative of MS18, it was tested for the presence of the amber suppressor. The recipient strain MS374 does have an amber suppressor, but it does not seem to interfere with the mapping of the cys-1 locus.

If the cys-1 mutation is an amber mutation and MS374 contains an amber suppressor, then no cys-1 recombinants are expected in a mating between MS810 and MS374. However, cys-1 mutant recombinants were produced. The fact that cys-1 mutant recombinants can be selected

TABLE 8.--Plaquing ability of the P22 phage amber mutant, 11c1, on S. pullorum and S. typhimurium strains.[a]

Strain of Cells in Overlay	Number of plaques[b]
S. typhimurium LT2 (su^+)	2.0×10^4
S. typhimurium 192 (su^-)	3.2×10^{10}
S. pullorum MS35	3.0×10^3
S. pullorum MS6	1.7×10^4
S. pullorum MS18	2.7×10^{10}
S. pullorum MS355	7.0×10^4
S. pullorum MS374	3.0×10^{10}
S. pullorum MS807, MS810 MS811, MS812	0
S. typhimurium SU694	0

[a]Procedure as described in Materials and Methods.

[b]Phage added to overlay at a titer of 3.2×10^{10} pfu.

indicates that the cys-1 reversion and the appearance of the amber suppressor were probably independent events. Further support for the inference that the cys-1 mutation is not an amber mutation comes from the observation that five independently isolated revertants of cys-1 did not contain amber suppressors (data not reported).

Since the donor MS812 was also a cysteine revertant, an attempt was made to infect this cell with the P22 amber mutant. When MS812 was mixed with phage 11c1, no plaques

were produced. This result was not expected, because a background level of Plaque production generally occurred even in a cell that was not able to suppress the amber mutation (see S. typhimurium LT2 su^+ in Table 8). Since the use of the P22 amber mutant was the only method available for determining the presence of the amber suppressor, P22 lysogenic MS812 could not be tested.

Conjunction in Salmonella pullorum

Analysis of the production of the F-trp donors.-- Recall that no P22 plaques were produced when S. pullorum donors carrying the F-trp particle were infected with the P22 amber mutant (Table 8). Since these donors were produced by mating S. typhimurium SU694 with a S. pullorum tryptophan mutant (Godfrey, Ph.D. dissertation, Michigan State University, East Lansing, 1969), the S. typhimurium strain SU694 was also tested. No plaques were produced on S. typhimurium SU694. A phage adsorption test (Table 9) showed that S. pullorum MS812 and S. typhimurium SU694 did not adsorb P22 phage to any appreciable extent; however, they did adsorb more phage than the cell without the adsorption site.

Since the cells that did not adsorb P22 were both trp mutants and donors, another trp mutant (MS355) and another donor strain (MS806) were tested for comparison. These cells were able to plaque P22, indicating the lack

TABLE 9.--Number of unadsorbed phage after various incubation periods at 37°C to allow for phage adsorption.

Strain of Cell	Time for Phage Adsorption	Titer of Unadsorbed Phage pfu/ml
S. typhimurium 192[a]	0	1.0×10^6
	5	5.2×10^4
	10	4.8×10^4
	15	2.0×10^4
	20	3.8×10^4
S. typhimurium LT2fer2[b]	0	2.4×10^6
	5	2.2×10^6
	10	2.4×10^6
	15	1.9×10^6
	20	1.0×10^6
S. pullorum MS812	0	8.0×10^5
	5	3.0×10^5
	10	1.2×10^5
	15	1.0×10^5
	20	1.0×10^5
S. typhimurium SU694	0	4.3×10^6
	5	4.7×10^6
	10	4.0×10^6
	15	2.2×10^6
	20	1.6×10^6

[a] S. typhimurium 192 is capable of suppressing the P22 amber mutation.

[b] S. typhimurium LT2fer2 is a strain of cell that does not have the P22 adsorption site.

of P22 adsorption was not a function of the trp mutation or of the donor trait.

If S. tpyhimurium SU694 and S. pullorum F-trp donors did not plaque P22 because they lacked the receptor site for P22, then the genetic determinants for an altered P22 receptor site may have been transferred from S. typhimurium to S. pullorum during the mating that produced the S. pullorum F-trp donor.

Mutants of S. typhimurium resistant to P22 because of alterations in cell wall synthesis involving the P22 receptor sites were reported by Mäkelä (76). Two mutations that result in altered phage receptor sites are the rough mutation, rouB, and the semirough mutation, rouC. These mutations in S. typhimurium map 10 minutes either side of trp. Since S. typhimurium SU694 is an F-trp secondary F prime, chromosomal as well as episomal genes may have been transferred during conjugation. The apparent lack of the receptor site in the S. pullorum F-trp donors suggest the idea of transfer of a rou mutation site.

If S. pullorum had received the gene for the rou mutation from S. typhimurium, more DNA was transferred than just the F-trp particle during the formation of the S. pullorum donor. The S. pullorum F-trp donor would be a hybrid containing chromosomal DNA from S. typhimurium.

The S. typhimurium SU694 and the S. pullorum F-trp donor cells were tested for the rough characteristic using the tester phage described by Gemski and Stocker (41). The tester phage growth pattern (Table 10) indicated that S. pullorum MS810 and S. typhimurium SU694 were not rough mutants, but smooth cells lysogenic for P22. The lysogeny of these cells clarifies why they would not plaque the P22 phage amber mutant, but is not the reason for the poor capacity of these cells to adsorb P22. Since there was some adsorption of P22, though poor, lysogeny seems a more adequate explanation for the inability of these cells to plaque P22 phage.

Probably the S. pullorum donors carrying F-trp became lysogenic for P22 when they were produced by matings with S. typhimurium SU694 either by transfer of P22 in the integrated state during conjugation, or more likely by infection with P22 phage subsequent to its production in the mating mixture by zygotic induction.

If it is true, as concluded above, that the lack of phage production in S. pullorum F-trp donors is due to lysogeny rather than to a rare mutation producing a mutant receptor site, then it can be concluded that the amount of DNA transferred from S. typhimurium to S. pullorum was restricted to the F-trp particle and did not include any chromosomal DNA.

TABLE 10.--Ability of various cell types to support the growth of several bacteriophage.[a]

Cell Type	Bacteriophage Type			
	Fo	Ffm	P22.c2	P221.c2
Standard Test Response				
Smooth lysogenic	+[b]	-	-	-
Smooth non-lysogenic	+	-	+	-
Rough lysogenic	-	+	-	-
Rough non-lysogenic	-	+	-	+
Response of Various *Salmonellae* Tested				
S. pullorum MS810	+	-	-	-
S. pullorum MS18	+	-	+	-
S. pullorum MS374	+	-	+	+
S. pullorum MS375	+	-	-	-
S. typhimurium SU694	+	-	-	-

[a]Test system is described by Gemski and Stocker (41).

[b]Indicates phage growth.

Recombination frequency in the S. pullorum conjugation system.--Comparison of recombination frequency for markers transferred in the S. pullorum conjugation system with the S. typhimurium and E. coli systems (26) shows that recombination frequencies for S. pullorum are lower.

After the discovery that the S. pullorum donor MS810 was lysogenic for P22 phage, the recipient MS374 was tested (Table 10) and found to be non-lysogenic. Matings between a lysogenic donor and a non-lysogenic recipient cell result in production of phage and lysis of some of the potential recombinants due to zygotic inducation of the lytic cycle of the phage (52, 114). On the other hand, when the donor strain is non-lysogenic and recipient is lysogenic, or when both parental types are lysogenic, no zygotic induction occurs.

An attempt was made to isolate a non-lysogenic donor by inducing the phage by growth at an elevated temperature. The cells were grown at temperatures up to 46°C but no non-lysogenic donor strains were produced. Since zygotic induction can also be prevented by the use of a lysogenic recipient cell in matings with the lysogenic donor, a lysogenic strain of MS374 was isolated. This cell, designated MS375, was used as a recipient in matings with the MS810 donor. Analysis of the recombination frequencies for the matings recorded in Table 11 indicate that recombination frequencies for all markers

TABLE 11.--Recombination frequency using lysogenic and non-lysogenic recipient types.

Mating Strains	Counter-selection	Marker Selected	Recombinants per Initial Donor Cell
MS810 x MS374	*his*-4	Pro^+	6.8×10^{-4}
		Ilv^+	1.8×10^{-4}
		Thr^+	6.3×10^{-5}
MS810 x MS375	*his*-4	Pro^+	1.5×10^{-2}
		Ilv^+	4.8×10^{-3}
		Thr^+	1.1×10^{-3}

are higher in this mating system when donor and recipient are both lysogenic. The recombination frequencies approach those reported for *S. typhimurium* (26).

Interrupted matings were done to establish the time of entry for *pro*, *ilv* and *thr* using this newly isolated lysogenic recipient MS375. The data from these matings are plotted in Fig. 1. Six experiments were performed and the data averaged and reported in Table 12. Proline enters at 39 minutes, isoleucine at 68 minutes, and threonine at 98 minutes. These values vary only slightly from those reported by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) using MS374 as a recipient, which indicates that lysogeny of these strains does not interfere with chromosome transfer and recombinant formation.

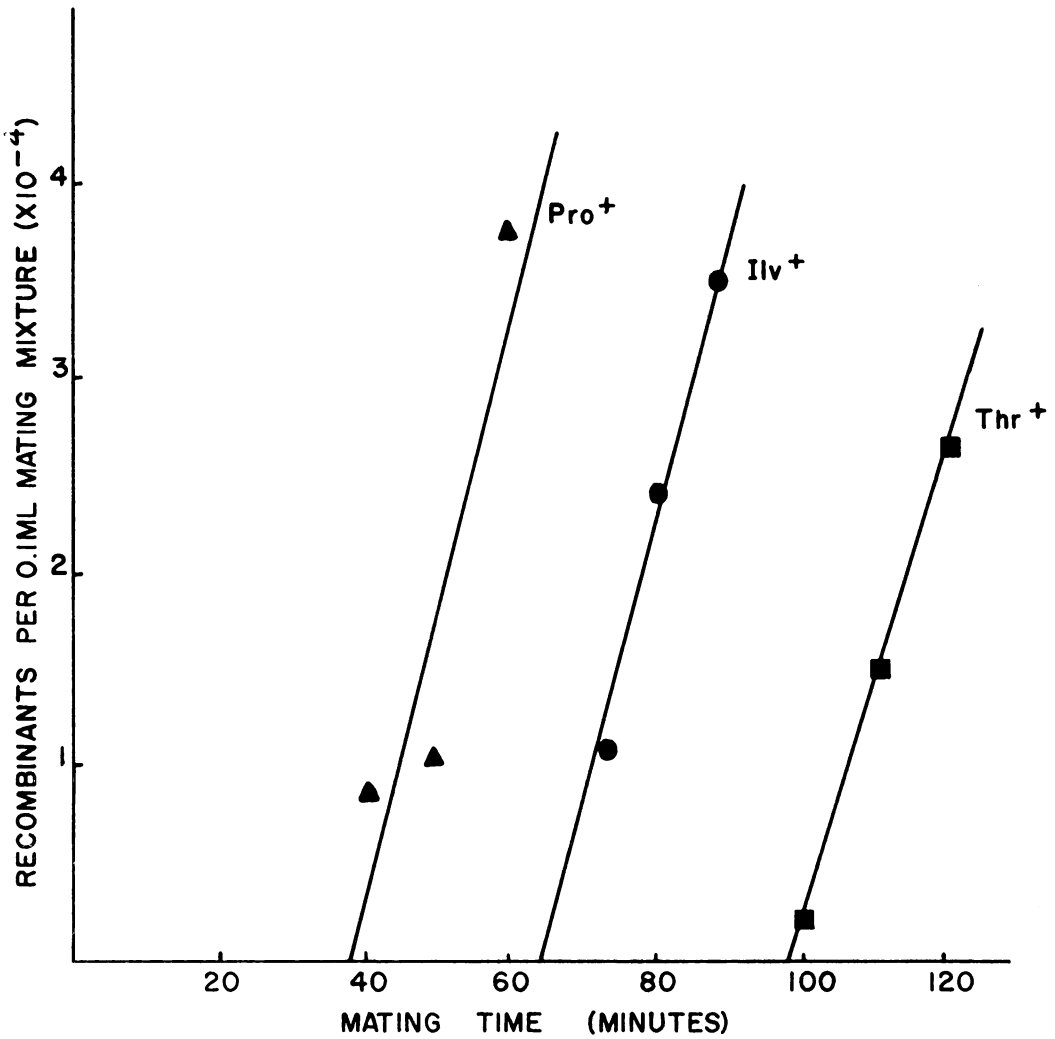

Figure 1.--Recombinants from interrupted conjugation, MS810 x MS375.

TABLE 12.--Time of entry for markers selected in matings between S. pullorum MS810 and MS375.

	Marker Selected		
	Pro^+	Ilv^+	Thr^+
Time of entry in minutes[a]	39	68	98

[a]Time given is an average from six experiments.

Excluding transformation and/or transduction as modes of marker transfer by MS810.--During the course of the conjugation studies using MS810 and MS374, two things were noted. First, phage were produced by zygotic induction; three types of phage were produced. Second, when the test for crossfeeding involved layers of donors and recipients in soft agar separated by a Millipore filter (.45μ), after 10 days of incubation, a few cells were growing on the plate above and below the filter disc.

These observations led to the inference that in S. pullorum matings, transformation and/or transduction may be accompanying conjugation. In some S. pullorum matings, the recombination frequency was so low that 1×10^9 to 1×10^{10} cells were plated in a single soft agar overlay. This large number of cells made it possible for transduction and/or transformation to occur even after the cells were plated.

Spontaneous transformation has been reported to occur by Ephrati-Elizer (38) and Ottolenghi-Nightingale (86). Though transformation has been studied primarily in Bacillus, it has been reported in E. coli (4). If transforming DNA is leaked into the medium by autolysis of donor cells in the mating mixture, then treatment of the mating mixture with DNase ought to abolish the transformation. A broth mating between MS810 and MS374 was performed in the presence of 20 µg/ml DNase. Matings of 10 minutes and 130 minutes showed no noticeable difference in the number of recombinants (Table 13). These results indicate that spontaneous transformation does not accompany conjugation in this system.

TABLE 13.--Recombinants per ml mating mixture in a broth mating with and without the addition of 20 µg/ml DNase.

Mating Strain	Time of Mating	Marker Selected	Number of Recombinants per ml Mating Mixture	
			Without DNase	With DNase
MS810 x MS374	10 min	Pro^+	2.1×10^3	2.2×10^3
	130 min	Pro^+	4.1×10^4	2.2×10^4
		Ilv^+	8.2×10^3	3.1×10^3
		Thr^+	2.9×10^3	7.5×10^3

Because phage are produced in the supernatant of a broth mating between MS810 and MS374, transduction may be accompanying conjugation. It may be argued that transduction cannot occur in this system because the cell in which the phage are produced by zygotic induction is auxotrophic for those markers which will be transduced. The result will be no transduction because the donor on which the phage is produced has exactly the same genetic defect as the recipient cell in the transduction.

However, this argument cannot be used to rule out the possibility of transduction, as Demerec (31, 32) reported transduction between homologous mutant cells giving rise to wild-type phenotype. Therefore, transduction remains a possibility in this system.

The first test for transduction involved treating the mating suprenatant with DNase and chloroform and then using it in an attempt to transduce Pro^+, Ilv^+ and Thr^+ in MS374. Examination of the plates used to select transductants seemed to indicate transduction had occurred (Table 14). However, when these "transductants" were streaked for isolation on media identical to that on which they were first selected, no growth occurred. If the cells that were growing on the original selection plates were transductants, they were abortive transductants. Though this test cannot be used to support the presence or absence of transduction, it seems unlikely

TABLE 14.--Test for the production of transductants with the recipient MS374 using the supernatant from a 7 hour broth mating between MS810 and MS374.[a]

	Marker Selected					
	Untreated Supernatant			DNase Treated Supernatant		
	Pro^+	Ilv^+	Thr^+	Pro^+	Ilv^+	Thr^+
Number of colonies growing per plate of selection media	400 802 721 353	384 390	260 440	726 430 916 393	874	466 530

[a]The supernatant contained 2.2×10^7 pfu/ml.

that transduction makes a major contribution to the number of recombinants for the following reasons. First, if self-transduction makes a major contribution to the number of recombinants produced, then recombinant production ought to be highest when the number of phage produced is greatest. The 50 fold increase in the number of recombinants produced when both donor and recipient are lysogenic (no zygotic induction of phage) suggests that the number of transductants among the recombinants is not major. Second, matings between MS810 and MS374 produce recombinants which show the inheritance of one or two unselected markers (Table 7) whose map positions are 40-50 minutes apart (Table 12). If transduction were responsible for the transfer of these markers, a double or triple infection

of the recipient is required--an extremely unlikely event. The linkage of markers in the recombinant is also inconsistent with self-transduction.

Characteristics of S. pullorum donors

The FT71 donor strain.--The S. pullorum donors carrying the FT71 factor were produced by transfer of the FT71 factor from S. typhimurium SU694 into a tryptophan mutant of S. pullorum (Godfrey, Ph.D. dissertation, Michigan State University, East Lansing, 1969). The genotype of this donor is expected to be trp^+ F / trp-2 with respect to the tryptophan genes. It is predicted that the Trp^+ marker will be transferred at a high frequency because of its attachment to the F-prime particle. When the transfer of the Trp^+ marker was compared to the transfer of the chromosomal marker Pro^+ (a marker transferred at 40 minutes), the Trp^+ was found to be transferred at a frequency of 8.8×10^{-4} per donor cell (Table 6), and Pro^+ was transferred at a frequency of 6.8×10^{-4} per donor cell (Table 11). These data show that Trp^+ is transferred at about the same frequency as Pro^+ is, and indicate Trp^+ is probably not being transferred as an independent F-trp unit.

One way to test for F-trp transfer is to examine the Trp^+ recombinant for the presence of the F function using MS2 phage. A test of the Trp^+ recombinants of an

FT71 donor mating for the presence of F showed that only 5% (21/447) were sensitive to MS2 phage. These observations indicate that the S. pullorum FT71 donor no longer carries the F-trp as an autonomous particle, but that the F-trp is probably integrated into the chromosome. Unusually stable integration of an F-prime into the chromosome has been reported by Jacob et al. (57) and Bergquist and Adelberg (11).

Additional support for this idea of F-trp integration into the chromosome comes from the following observations made during the course of this study. The donor cells used in mating were selected as single cell isolates from L agar plates. It is sometimes found that cells carrying F-prime particles tend to lose them and segregate cells that no longer carry these particles. However, every isolate of S. pullorum MS810 picked for 88 separate mating experiments performed over a period of 2 years, was Trp^+ and donated its chromosome to recipient cells. It was predicted that if the FT71 carrying donor had the genetype trp^+ F / trp-2, some of the recombinants would be trp mutants. Analysis of recombinants for Trp^-, as an unselected marker, showed that all recombinants were Trp^+.

F-prime particles are able to be cured from the cells containing them by treating the cells with curing agents such as ethidium bromide (12). Cells of S. pullorum

carrying FT71 were treated with EtBr and tested to see if any Trp^- cells were produced. Out of 1130 cells examined, no Trp^- cells were found. Some of the treated cells were also tested for the presence of the F particle using MS2 phage. An F^-, Trp^+ cell was isolated from curing experiments.

A cell with a genotype similar to that of the S. pullorum FT71 donor strain produced by Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) was constructed and tested to determine the relationship between the F-trp particle and the chromosome. S. typhimurium SU694, a cell carrying the FT71 F-trp particle, was mated with S. pullorum MS103, a cysB1 trp-3 mutant. From this mating, 64 Trp^+ recombinants were selected and tested for the presence of the F-trp using MS2 phage. All 64 isolates were MS2 resistant, indicating that the Trp^+ marker is being transferred from SU694 as a chromosomal rather than an F-prime marker. Sanderson and Hall (97) reported the frequency of chromosome mobilization in S. typhimurium F-trp is as much as 30 times higher than the F-trp transfer.

At the same time the SU964 x MS103 mating was performed, the S. pullorum F-trp donor MS810 was mated with MS103. From this mating, 40 Trp^+ recombinants were selected and tested for MS2 sensitivity. Two of the recombinants were MS2 sensitive. The fact that most of

the Trp^+ recombinants were not MS2 sensitive indicates that in S. pullorum MS810 the trp^+ allele is primarily transferred as a donor chromosomal allele rather than an F-prime donor allele.

One of the Trp^+ isolates from the MS810 x MS103 mating that was MS2 sensitive was grown in the presence of EtBr in an attempt to cure the Trp^+ characteristic. None of the 662 isolates tested was Trp^- and of 60 isolates tested for MS2 sensitivity, all were MS2 sensitive.

All these findings indicate that in S. pullorum donors carrying FT71, a recombination has occurred which produced a homogenote ($trp\text{-}2^+/F\ trp^+$) for the loci carried on the F-prime. This integration of the F-prime accounts for the fact that Trp^+ recombinants are generally F^- (MS2 resistant). These observations on S. pullorum donors carrying the FT71 indicate that the F-trp in S. pullorum acts very much as it does as a secondary F-prime in S. typhimurium (97). Both strains show more frequent transfer of the chromosome than the intact F-prime, and in both cases, Trp^+ recombinants are generally F^-.

S. pullorum Lac^+ donors.--A Trp^+ MS2 phage resistant S. pullorum (MS900) cell was isolated following treatment of MS810 with EtBr. This cell had no donor ability when mated with S. pullorum recipients. MS900 either may have lost its F particle but, because of a prior recombination

event, remained Trp^+, or may have been produced by a mutation in F whose minimum functional consequence is the loss of F pili production. Examination of MS900 using the electron microscope showed that it did not produce F-pili.

If the cell is MS2 resistant because it has lost the entire F particle, then the cell can again accept an F particle and become a donor. If, however, the cell is a mutant of F that has lost its ability to produce F pili, then introduction of a new F particle can lead to the exclusion of this new F particle by the mutant F still residing in the cell (36, 78, 98).

In order to determine if a newly added F can be accepted by this MS2 resistant cell, it was mated with an *E. coli* AB785 F-*lac* donor. The F-*lac* factor was chosen as the particle to introduce because *S. pullorum* like *S.* typhimurium, does not apparently carry any lactose genes, and Lac^+ *S. pullorum* recombinants indicate a successful gene transfer from *E. coli* to *S. pullorum*. Among the Lac^+ *S. pullorum* recombinants, F-*lac* *S. pullorum* cells are expected.

The MS2 resistant Trp^+ cell (MS900) and MS103 were mated with *E. coli* AB785. $Lactose^+$ *S. pullorum* recombinants were isolated and tested for MS2 sensitivity. It was difficult to isolate Lac^+ *S. pullorum* cells even when conditions were varied in an attempt to increase recombinant production. The Lac^+ isolates from the

AB785 x MS900 mating were all MS2 sensitive; whereas the Lac^+ isolates from the MS103 mating, $MS103L^+$, were all MS2 resistant.

The Lac^+ S. pullorum cells were treated with EtBr to determine if the Lac^+ characteristic was curable. The data from these experiments (Table 15) indicated that Lac^+ was easily cured in MS920, but in $MS103L^+$, it was not cured to any appreciable extent.

TABLE 15.--Curability of Lac^+ in E. coli and S. pullorum with ethidium bromide.

Cell Tested	EtBr	Number of Cells		Percent Lac^-
		Lac^+	Lac^-	
E. coli	-	2055	15	1
AB785	+	1048	51	5
S. pullorum	-	398	20	5
$MS103L^+$	+	1448	67	5
S. pullorum	-	355	1	0.5
MS920	+	27	1159	98
S. pullorum	-	350	0	0
MS921	+	321	0	0

The lack of curing in E. coli AB785 treated with EtBr may be due to the fact that in this cell the F-lac

exists predominantly in the integrated state and not as an autonomous particle. If the lac gene is integrated into the chromosome of E. coli AB785, the Lac^+ marker can be introduced into the S. pullorum recipient either as an episomal or a chromosomal marker. If Lac^+ is an episomal marker, the recombinant will likely be F^+; and if Lac^+ is a chromosomal marker, the recombinant will most likely be F^-. It appears that MS920 and MS921 received Lac^+ as an episomal marker; whereas, $MS103L^+$ received Lac^+ as a chromosomal marker or if it received F-lac, the lac gene is integrated and F excluded.

The difficulty encounted in isolating Lac^+ S. pullorum cells following a mating between AB785 and MS900 does not preclude the presence of some mutant F in MS900, however, as it was just as difficult to isolate Lac^+ S. pullorum cells from a mating using the S. pullorum recipient strain MS103.

Mating experiments were set up between MS920 and MS375 to test the donor ability of MS920. The data from Table 16 and Figure 2 indicate that MS920 was able to mobilize the S. pullorum chromosome in a manner that resembled the mobilization produced by its progenitor MS810.

These data can be interpreted as follows. The alteration of F produced by EtBr treatment results in

TABLE 16.--Recombination frequency from a three-hour broth mating.

Mating Type	Marker Selected	Recombinants per Initial Donor Cell
MS920 x MS375	Pro^+	1.1×10^{-2}
(First mating)	Ilv^+	2.1×10^{-3}
	Thr^+	2.5×10^{-4}
MS920 x MS375	Pro^+	2.8×10^{-4}
(Second mating)	Ilv^+	4.3×10^{-4}
	Thr^+	1.5×10^{-4}

the loss of the pilus-producing function of F and a modification of its ability to exclude another F. The correction of the pilus-producing function of F, by complementation from the autonomous F-lac, restores the donor ability of the cell, with chromosome mobilization being controlled by the remaining portion of the original F-trp particle. It is possible that most of the functions of F were performed by the F-lac. The only function of the integrated F that it necessarily would have to perform is to provide the site for the action of the nuclease which produces the single-stranded break necessary for the linearization of the chromosome. The site of this break determines the oirgin of transfer for the chromosome (11, 57).

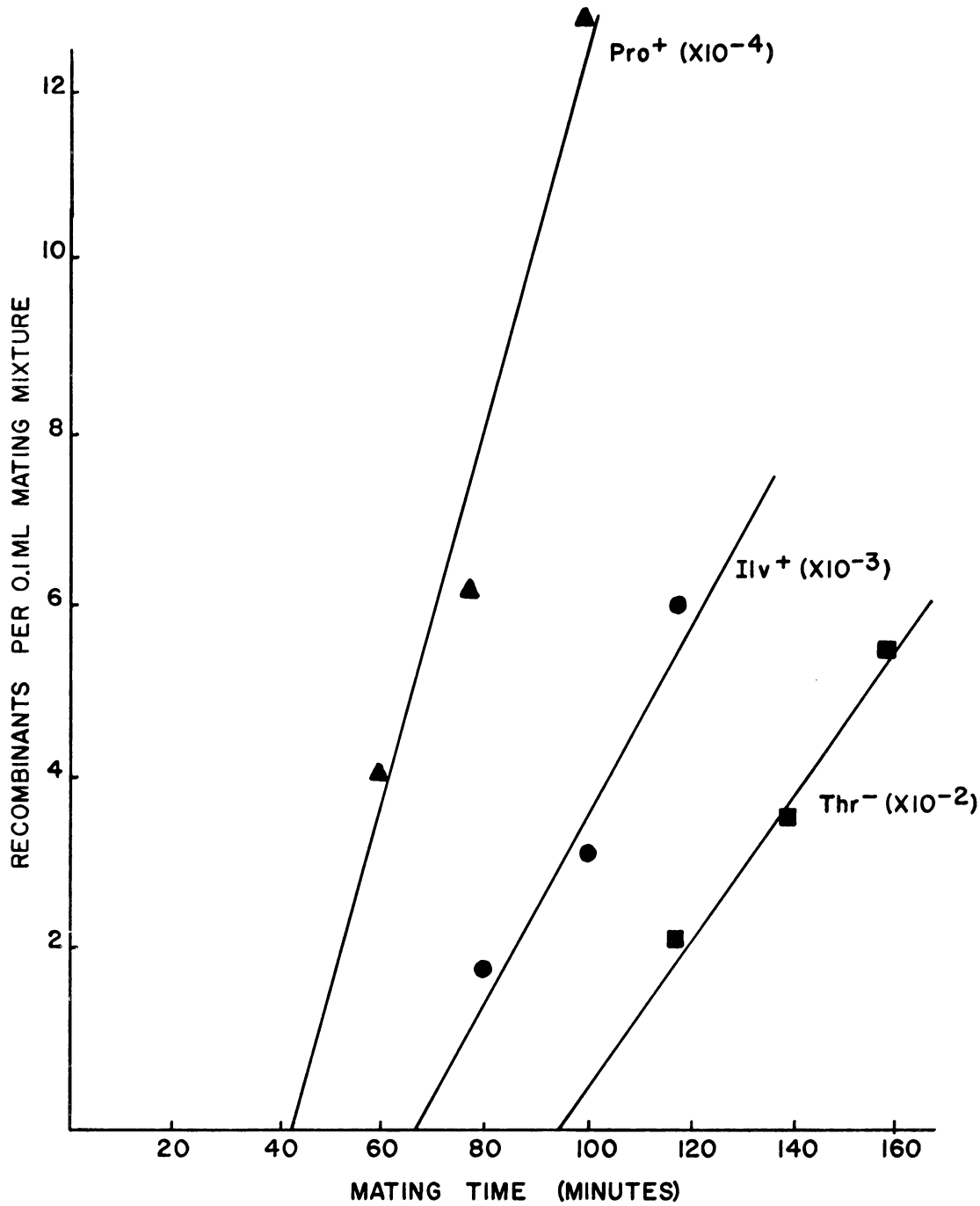

Figure 2.--Recombinants from interrupted conjugation, MS920 x MS375.

When selection for unselected marker studies were performed on the recombinants from the first mating between MS920 and MS375, it was noted that a number of recombinants were unstable, segregating cells that had lost the donor marker. This characteristic was particularly noticeable when Pro^+ recombinants were selected.

Recombinants from MS920 x MS375 were analyzed for Lac^+ as an unselected marker. The fact that Lac^+ cells were not found indicated that F-*lac* was not transferred simultaneously with the chromosome. This finding is in agreement with that reported for *S. typhimurium* by Sanderson and Hall (97) in which they state that there is no detectable simultaneous transfer of F-prime and the chromosome.

Analysis of the unselected marker data recorded in Table 17 indicates that MS920 did not behave exactly as its pregenitor MS810. The high linkage of all markers and lack of stable inheritance of donor markers are interpreted to mean that the DNA introduced into the recipient cell is not undergoing normal recombination but is maintained in the autonomous state. An indication that this modification in the recombinant production is a function of the mutant F is that the modification appears only when MS920 is the donor and not when MS810 or MS921 (see following section) are used as donors.

TABLE 17.--Linkage analysis of recombinants from a three-hour broth mating.[a]

Unselected Marker	Selected Marker		
	Pro^+ 74[b]	Ilv^+ 105	Thr^+ 106
Pro^+	-	99%	75%
Ilv^+	93%	-	100%
Thr^+	0	98%	-
Str^+	-[c]	24%	52%
Gal^+	5%	51%	3%
Lac^+	0	0	0

[a]Data from a mating between MS920 and MS375.

[b]Number of recombinants tested.

[c]Data not available.

A second mating was performed using the MS920 donor. Recombination frequency data (Table 16) showed that the MS920 strain was losing its donor ability. When tested, this cell line appeared to be segregating large numbers of Lac^- cells with no donor ability, and as a result, the MS920 donor strain was lost. The loss of F-*lac* by MS920 is probably due to a delay in the expression of F incompatibility in this strain.

In an attempt to recover the MS920 strain, another Lac^+ MS2 sensitive cell (MS921) was isolated following a mating between *E. coli* AB785 and MS900.

When MS921 was grown in the presence of EtBr it was found that the Lac^+ characteristic was not curable (Table 15). The lack of curability in MS921 indicates that the new isolate, MS921, differs from the first Lac^+ MS2 sensitive isolate, MS920, in that the F-lac appears to be integrated into the chromosome. The integration of F-lac in MS921 was not forced since there was no selection pressure for the production of a Lac^+ cell. Because the MS900 cell most probably retains at least some integrated F DNA, the MS921 cell line may have escaped the incompatability of the presence of two F factors by having them both integrated into the chromosome as suggested by Dubnau and Maas (36).

The donor ability of MS921 was tested by mating it with MS375. The production of Pro^+, Ilv^+ and Thr^+ recombinants indicate that MS921 is able to transfer its chromosome to a recipient cell.

In matings between MS921 and MS375, the mating mixture was plated as usual in E-minimal soft agar over a base layer of media selective for Pro^+, Ilv^+ or Thr^+ recombinants. After incubation for 3 to 4 days, an anomaly was noted. On plates selective for Thr^+ recombinants, the plates made from the undiluted mating mixture showed a lower number of presumptive recombinants growing than did the plates with mating mixture diluted 1/10. This phenomenon was observed several times. In another

mating system (Pseudomonas aeruginosa) anomalous behavior of the type described above was also reported (71).

The basic difference between these two types of plates, in addition to the different number of cells, was that the plates with undiluted mating mixture had 0.1 ml of L broth in the overlay, whereas the plates with diluted mating mixture had only 0.01 ml L broth. The mating mixture was routinely diluted in E minimal medium prior to plating in E minimal soft agar.

In order to eliminate the L broth, a Millipore filter mating was done and the mated cells were resuspended in E minimal medium. Using this technique, there was a better correlation between dilution and the number of recombinants produced on the plate.

Prolonged (3 hour) matings between MS921 and MS375 were performed and the data recorded in Table 18. From these data it appears that the order of entry for markers using the MS921 donor is opposite that produced by the MS810 donor. This reversed order could indicate that the chromosome is being mobilized in the opposite direction.

In an attempt to confirm the predicted order of entry produced by the MS921 donor, recombinants were selected and analyzed for the presence of unselected markers. These data (Table 19) do not unequivocally support the order of entry predicted from the recombination frequency.

TABLE 18.--Recombination frequency from a three-hour broth mating.[a]

Mating Type	Marker Selected	Recombinants per Initial Donor Cell
MS921 x MS375	Thr^+	1.1×10^{-2}
	Ilv^+	2.1×10^{-3}
	Pro^+	1.5×10^{-4}

[a]The data reported is an average calculated from four separate mating experiments.

TABLE 19.--Linkage analysis of recombinants from a three-hour broth mating.[a]

Unselected Marker	Selected Marker		
	Thr^+ 199[b]	Ilv^+ 296	Pro^+ 281
Thr^+	-	40%	51%
Ilv^+	1.5%	-	24%
Pro^+	2.0%	18%	-
Lac^+	47.0%	58%	52%
Gal^+	1.8%	5%	31%
Str^+	2.5%	8%	4%

[a]Data from a mating between MS921 and MS375.

[b]Number of recombinants tested.

Recombinants for Pro^+, Ilv^+ and Thr^+ were also tested for the presence of Lac^+ as an unselected marker. Approximately 50% of all recombinants received Lac^+. When these Lac^+ cells were tested with MS2 phage, some of the Lac^+ cells were MS2 sensitive. These data indicate that Lac^+ is transferred as an early chromosomal marker rather than as an episomal marker. These data, along with that on EtBr curing, support the idea that the F-lac in MS921 has been rather stably integrated into the S. pullorum chromosome. Scaife and Gross (99) also reported transfer of Lac^+ as an early marker in mobilization produced by an F-lac but in E. coli.

At this point in our study, the following explanation for the donor ability of MS921 was constructed. In MS921, chromosome mobilization is produced by integration of F-lac into the chromosome according to the model proposed by Scaife and Gross (99) for mobilization by F-prime factors. Since S. pullorum does not have any lactose genes, unless they are cryptic, the F-lac cannot be integrated due to lactose homology. Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) reported that S. pullorum does not naturally have any regions of F homology to provide for integration. The cell into which the F-lac was introduced, MS900, probably has some F DNA remaining in the trp region of the chromosome and this region of homology is responsible

for the integration of F-lac. If the F-lac is integrated into the chromosome with the opposite orientation from that of the original F-trp, then the chromosome is mobilized in the opposite direction.

Data from interrupted matings (Figure 3) did not support the proposed model, namely that chromosome transfer in MS921 is from a single origin in one direction only. These results are similar to those reported for F^+ transfer, in which there is no specific single point of origin.

Since S. pullorum has not been shown to have F homology or lac homology, it appears that either integration of F-lac is not necessary to produce chromosome mobilization or homology is not necessary for F-lac integration. It is hypothesized that the random nature of chromosomal transfer by MS921 is the result of an alteration in the specificity of the nuclease responsible for the linearization of the chromosome (11, 57). If the nuclease loses its specificity for the integrated F, linearization of the chromosome and subsequent transfer can begin at any point.

The fact that Lac^+ was transferred as an early marker to about 50% of the recombinants indicates that the chromosome transfer is produced by the integration of F-lac. It appears that the integration of F-lac can

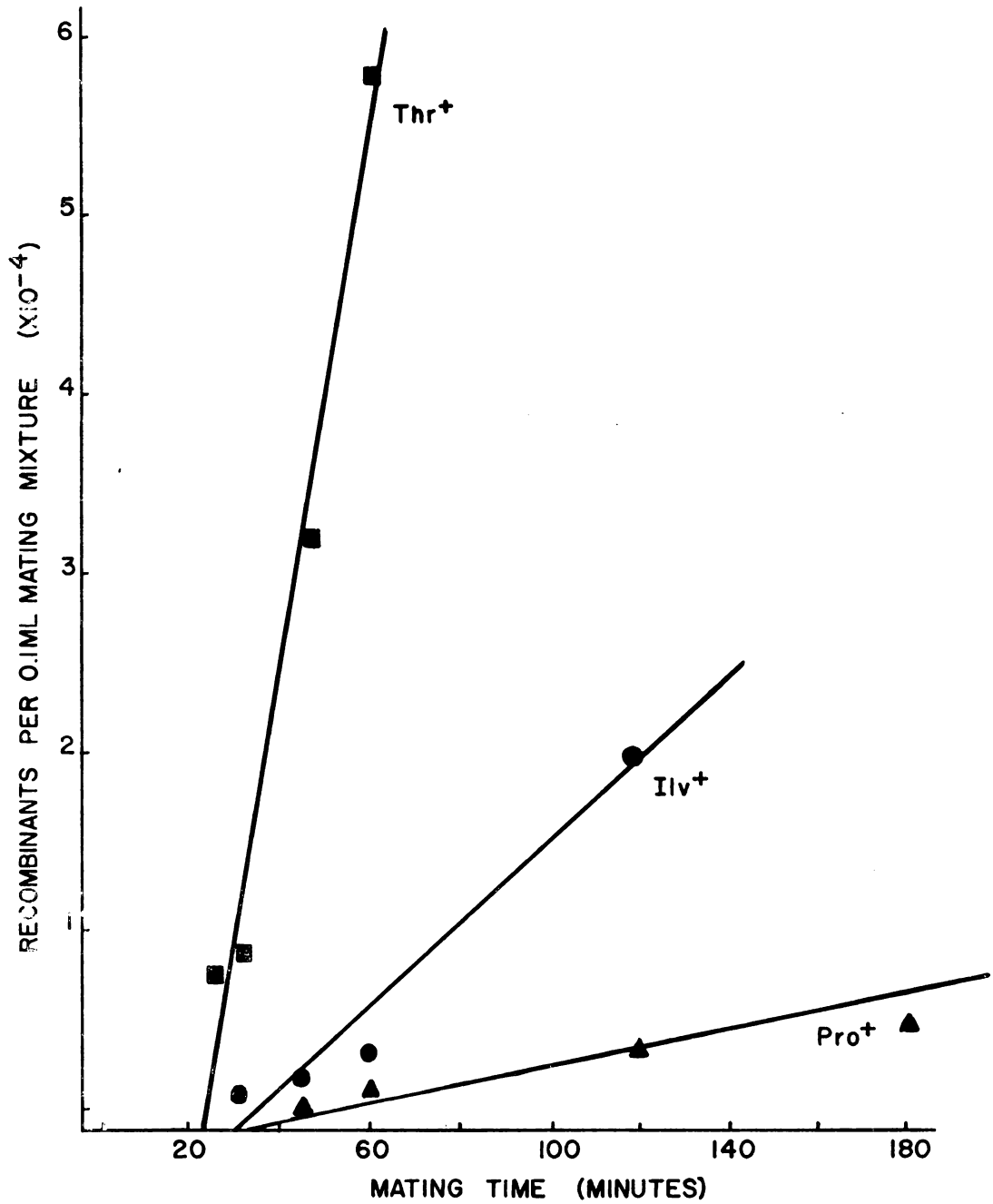

Figure 3.--Recombinants from interrupted conjugation, MS921 x MS375.

occur randomly along the chromosome, though possibly at higher frequency in some locations.

All markers show about the same delay in transfer over that produced by F^+ type transfer. This delay may reflect the amount of time necessary for integration and transfer of F-*lac* at a lead position on the chromsome (90).

The *S. pullorum* Linkage Map

A linkage map for *S. pullorum* was constructed using data from Tables 7, 11 and 12 (Figure 4). This linkage map for *S. pullorum* resembles grossly the linkage map of *S. typhimurium* presented by Sanderson (96). These similarities are to be expected since the two species are related and display certain common properties. In addition, these two species were reported (D. E. Schoenhard, Bacteriol. Proc., p. 30, 1963) to have fine structure homology.

In the linkage map, one of the differences between *S. typhimurium* and *S. pullorum* involves the threonine region. Godfrey (Ph.D. dissertation, Michigan State University, East Lansing, 1969) reported that in *S. pullorum* the *thr* region is both inverted and transposed with respect to its position in *S. typhimurium*. He also reported that very few recombinants are produced in a mating between an *S. typhimurium* donor and an *S. pullorum*

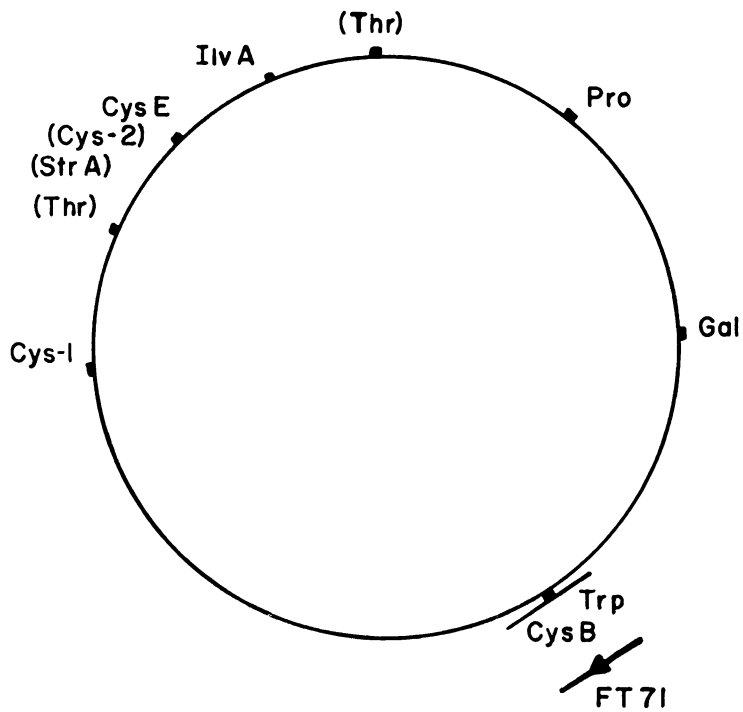

Figure 4.--Linkage map of Salmonella pullorum. Markers given in parenthesis are not precisely located but only placed in approximate positions.

recipient when the origin of mobilization in the donor is near the thr region.

According to the linkage map constructed by Godfrey and supported by some of the work reported here, one would expect more Ilv$^+$ recombinants than Thr$^+$ recombinants in matings using FT71 carrying donor strains. When the number of recombinants per ml of mating mixture was determined, generally there were more Ilv$^+$ recombinants. However, occasionally in a mating, the number of Thr$^+$ recombinants was equal to or slightly higher than the number of Ilv recombinants.

Other irregularities were found with respect to the thr region. The Thr$^+$ recombinants were more difficult to select and purify than the Pro$^+$ or Ilv$^+$ recombinants. Occasionally linkage studies would indicate a higher linkage of thr to pro than to ilv. In addition, the gene order of thr ilv cysE based on linkage analysis of recombinants from an MS812 x MS91 mating was o-thr--ilv--cysE when Thr$^+$ was selected but o-ilv--thr--cysE when Cys$^+$ or Ilv$^+$ recombinants were selected.

The difficulty in positioning the thr locus on the genetic map may be related to the transposition of threonine. There may be two regions of the threonine homology. If the thr region was originally found between ilv and pro where it is in S. typhimurium, then its transposition could have involved a duplication of the

thr region. According to Campbell's model for excising a loop of DNA, homology of two regions is a necessary prerequisite. If the excised loop of DNA reintegrated into the chromosome at another location with the orientation of the loop reversed, an inversion and transposition would result. Another possible consequence of this type of arrangement is two separate regions with partial threonine homology, one in the original position with the original orientation and the second inverted and transposed. If the threonine region were so constructed, these alterations would account for the problems associated with experiments which attempt to establish the position of thr on the genetic map.

Because of the difficulty associated with the establishment of the position for the thr locus, the exact location of str-1 and cysE cannot be definitely established. The cysE and str-1 loci may be located in S. pullorum where they are in S. typhimurium but with the mutants available the precise location cannot be established.

Repeated attempts were made to introduce additional mutations on several recipient cells. For example pro or trp on MS91, trp on MS374, pro, ilv or thr on MS105. When NTG treatment did not allow for the selection of the desired mutants, a nitrous acid mutagenesis was attempted without success. It appears

as though the cell can be mutated only to a certain extent and that attempts to add the desired mutations are not successful either because of lethal effects or because the mutations produced are not the ones being selected.

SUMMARY AND CONCLUSIONS

Mutants of S. pullorum were isolated following NTG treatment. The cysteine mutants were placed into four groups by auxanographic testing. Mutants representative of these four groups were Millipore mated and recombinants selected. Recombination frequency and linkage analysis of these recombinants support the conclusion that in S. pullorum these four cysteine genes are scattered along the chromosome rather than being located in a single locus or closely spaced loci.

Experiments were performed to demonstrate that marker transfer in the S. pullorum transfer system was conjugal and that the contribution of transductants or transformants to the total number of recombinants was lacking or very small. The co-inheritance in the recombinants of markers more than 40 minutes apart on the chromosome supports the conclusion that transduction and/or transformation do not make a major contribution to the production of recombinants. Marker transfer in the presence of DNase also supports the conclusions that transformation is not a major contributor to the production of recombinants.

Analysis of CysB$^+$ and Trp$^+$ recombinants from broth and Millipore matings using the F-trp S. pullorum donor MS810 indicates that this donor has the F-trp factor rather stably integrated into the chromosome to produce an Hfr type donor. The transfer of a terminal chromosomal marker by this donor in a 3 hour mating indicates transfer of the entire chromosome. Since cysB$^+$ and Trp$^+$ are co-transducible, the transfer by MS810 of Trp$^+$ as a proximal marker and CysB$^+$ as a terminal marker indicates the circular nature of the S. pullorum chromosome.

Following treatment of the S. pullorum donor MS810 with ethidium bromide, an MS2 phage-resistant isolate, MS900, was selected. Examination of MS900 with the electron microscope showed it lacked F-pili. From a mating between MS900 and E. coli AB785 two different Lac$^+$ isolates were selected. In one isolate, MS920, the F-lac appears to remain autonomous, restoring the donor ability by complementation of the missing function(s) of F. The MS920 cell type appears to donate its chromosome as its progenitor, MS810. The second isolate, MS921, appears to have the F-lac factor stably integrated into the chromosome. The MS921 donor produces an unusually high-frequency, random transfer of the chromosome.

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