GENETIC AND PHYSICAL PROPERTIES OF F77 IN SALMONELLA PULLORUM MS35

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ABSTRACT

GENETIC AND PHYSICAL PROPERTIES OF
F77 IN *SALMONELLA PULLORUM* MS35

By

Paul W. Stiffler

Recent conjugation experiments with *Salmonella pullorum* indicated that the F-prime factor F-*cysE*$^+$ *rfa*$^-$ *pyrE*$^+$ (F77) isolated from *Salmonella typhimurium* transfers the *S. pullorum* chromosome from one origin in two directions (42). This origin was different than the origin in *S. typhimurium* from which F77 transfers only in the clockwise direction. Therefore, experiments were performed to investigate and compare the genetic and physical properties of F77 in *S. pullorum* and *S. typhimurium*.

In this study, however, the F-prime factor, F77, was found to transfer the *S. pullorum* chromosome from two different origins, both in the clockwise direction. The genetic loci studied appeared to be in the same relative position as they are in *S. typhimurium*. The primary origin of transfer was from *cysE* as 0-*cysE*-*ilv*-*thr*-*pro*. The secondary origin of transfer was from a locus between *pyrD* and *trp* as 0-*trp*-*cysB*-*his*. The recombination

frequencies for selected markers transferred from the secondary origin were 10 to 100 fold less than for selected markers transferred from the primary origin. The _trp cysB_ genes appeared to be inversed compared to those in _S. typhimurium_. The reduction in transfer of intact F77 factors, the increase in recombination frequencies of selected donor recombinants, and the stability of donor ability suggested that F77 converted from the autonomous state to a stable association with the donor chromosome. The donor carrying a spontaneous mutation in the cysteine gene of F77, designated F77_cysE_$^{-}$, transferred only from the secondary origin between _pyrD_ and _trp_ as O-_trp_-_cysB_-_his_, with the same relative frequency as F77. This strain also displayed extreme stability of donor ability.

Since the overall recombination frequencies for selected markers were 10 to 100 fold lower than expected for F-prime mediated chromosomal transfer, an experiment was designed to select a donor with increased transfer ability. It appeared that _S. pullorum_ donors carrying F77 were homogeneous with regard to F77, suggesting that F77 was able to transfer the host chromosome from either origin of transfer.

Electron micrographs of the _S. pullorum_ recipient showed no unusual surface structures while the _S. pullorum_ donors appeared to have at least 15 sex-pili per bacterium.

Paul W. Stiffler

The physical basis for the stability of F77 and F77*cysE*$^-$ in *Salmonella pullorum* was determined. The F77 factor was isolated in the autonomous state from the donor MS8300. F77 was no longer autonomous in a derivative of MS8300, designated MS830, which transferred the chromosome at a higher frequency. The F77*cysE*$^-$ in *S. pullorum* MS831 and F77 in *S. typhimurium* SA532 were isolated from the autonomous state. In *S. pullorum* MS830, F77 appeared to exclude the P0-2-like plasmid molecule, not phage P35, while F77*cysE*$^-$ and phage P35 in MS831 did not. Neither F77 nor F77*cysE*$^-$ nor phage P35 excluded the PO-1 plasmid molecule. The F77 and F77*cysE*$^-$ factors appeared to have sedimentation coefficients of 70s and molecular weights of approximately 51 x 10^6 daltons. It was concluded that F77 forms a very stable association with the chromosome of *S. pullorum*, while F77*cysE*$^-$ does not.

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Paul W. Stiffler

A THESIS

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This thesis is dedicated to

my wife Lois

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INTRODUCTION

A conjugation system for *Salmonella pullorum* has been elucidated by Godfrey (42). His data suggest that the *S. pullorum* chromosome exists as a single closed circular linkage map very similar to the linkage map of *Salmonella typhimurium*. The only obvious differences are an inversion of the *trp cysB* genes and a possible transposition of the *thr* gene.

Godfrey (42) observed that the *S. pullorum* donor MS809 carrying F77, an F-prime sex factor isolated from *S. typhimurium* carrying the *S. typhimurium* chromosomal genes *cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (K. E. Sanderson and Y. A. Saeed, personal communication), appears to transfer the host chromosome from one origin in two directions. This origin is not as reported in *S. typhimurium* where F77 transfers the host chromosome in only the clockwise direction (K. E. Sanderson and Y. A. Saeed, personal communication).

Therefore, it was decided that the biological and physical properties of F77 in *S. pullorum* and *S. typhimurium* should be investigated and compared.

LITERATURE REVIEW

Part 1

Conjugation

The process of bacterial conjugation was discovered and described by Lederberg and Tatum in 1946 (59, 60). This process is responsible for the unidirectional transfer of genetic material which occurs upon cellular contact between two bacterial cells of opposite mating types. The cell donating its genetic material contains a fertility factor termed F (21, 58). The recipient cell lacks F and is termed F^-. During conjugation there is a low frequency of mobilization of chromosomal markers (10^{-4} to 10^{-5}) but a high frequency of F transfer (.5 to 1) to F^- recipient cells (5, 29, 46, 58). Other genetic elements exist which can transfer themselves infectiously and promote chromosome-transfer such as colicinogenic (Col) and resistance transfer (R) factors (26).

Fertility factor (F). The F factor is an antonomous, covalently closed double strand DNA molecule (35, 36, 94) with an estimated molecular weight of 45 x 10^6 daltons (35, 37). The F^+ factor gene load is approximately 2% of the total bacterial genome and is large enough to carry approximately 100 genes (99). Among the known functions coded for by F genes are: sexual transfer or conjugal fertility (45, 46, 58, 71, 99) sex factor replication (66, 99), superinfectious immunity (64, 99), F-pilus formation (13, 14, 71, 93), growth inhibition of certain phages (71), f^+ antigen (74), and receptor sites for a group of male-specific phages (13, 14, 20, 26, 27, 67, 93, 99).

Rupp and Ihler (82), Ohki and Tomizawa (69), and Ihler and Rupp (50) inferred that the unique labeled strand of Hfr or F-prime DNA transferred during conjugation is due to the asymmetric transfer of a specific strand of sex-factor DNA with a 5' nucleotide at the origin of transfer. Vapnek and Rupp (94) conclusively showed that only the denser strand of the sex factor DNA with a 5' end at the origin, is transferred to the recipient where its complementary strand is synthesized resulting in a covalently closed sex factor DNA molecule. These results show that DNA synthesis associated with mating occurs in both the donor and recipient cells (94). The rolling circle model of DNA synthesis (39) provides a working model for this asymmetric strand distribution. The sex-factor strand that is not transferred to the recipient during conjugation synthesizes its complementary strand in the donor during mating and also forms a covalently closed double-stranded molecule (94).

Electron microscopy. The electron microscope is a valuable tool for visualizing the sex-pili (F-pili) formed on bacterial cells harboring the sex factor (F). Typical *Salmonella typhimurium* and *Escherichia coli* donors have 2-3 F-pili per cell. Donor specific DNA and RNA phage "stain" the F-pili by adsorbing to them (14, 27, 93). The DNA donor specific phage adsorb to the tip of the F-pili (20, 67) and the RNA donor specific phage adsorb to the sides of the F-pili (14, 20, 27, 67). The recipient cells have no sex-pili and therefore do not adsorb donor specific phage.

High frequency recombination donors (Hfr). Integration of the F^+ factor into the bacterial chromosome results in an Hfr strain (46) which transfers its chromosome in a specifically oriented and linear way. Due to random separation of the mating pairs the frequency of inheritance of donor markers in recombinants is highest if the marker is located near the beginning of the Hfr chromosome and lowest if it is near the end of the Hfr chromosome with the sex factor always the last marker to be transferred (47, 54). Early markers are transferred at a frequency of 10^{-1} to 10^{-2}. Integration of F^+ into the bacterial chromosome is believed to occur by reciprocal crossover between the circular F^+ factor and the circular bacterial chromosome resulting in a linear insertion of the F^+ factor into the bacterial chromosome (18). The bacterial chromosome remains circular, only slightly larger due to the presence of the integrated F^+ factor.

Curtiss and Renshaw (29) described two classes of F^+ donors according to their ability to give rise to stable Hfr derivatives. Type II F^+ donors fail to give rise to detectable frequencies of stable Hfr derivatives because the association between F and the chromosome is transient. They suggested three possibilities that may be responsible for the existence of Type II F^+ donor strains. First, stable F integration may not occur either because a bacterial enzyme(s) specifically necessary for F integration is absent or because the F attachment site on the membrane is altered, which in effect prevents pairing and/or exchange between F and the chromosome. Second, stable F integration may occur without causing the expression of the usual Hfr phenotype. Third, stable F integration resulting in chromosome lethality may occur because of the absence of a functional bacterial enzyme(s) necessary for complete integration of F into the chromosome or for the circularization of the F episome.

Intermediate donors (F-prime). Abnormal detachment of F from the Hfr state results in the removal of a segment of the bacterial chromosome with it. These sex factors are called F-prime factors and can transfer the chromosome at a high frequency and with the same orientation as the parental Hfr strain (3, 51).

Broda *et al.* (16) and Scaife (85) proposed that F-prime factor formation is the result of a reciprocal crossover between two chromosomal sites on either side of the integrated F or between a site in the integrated F and the chromosome. This model is essentially the reverse of Campbell's Model (18) of episome integration. A reciprocal crossover between two chromosomal sites results in an F-prime factor carrying segments from both distal and proximal regions of the ancestral Hfr chromosome (16). A reciprocal crossover between the chromosome and integrated F factor results in F prime factors carrying segments of the Hfr chromosome that are transferred proximally (62) or distally (11, 51, 62).

Part II

F-prime Donor Strains

Primary F-prime donors. The data of genetic experiments demonstrate that cells in which F-prime factors arise are haploid and the genes deleted from the chromosome are now on the F-prime factor (11, 78, 85, 87). These F-prime donors are designated as primary F-prime donors (11) and they transfer the F-prime factor to F^- recipients at nearly 100% efficiency but chromosomal genes not carried on the F-prime factor are transferred at random with frequencies of 10^{-4} to 10^{-5} per donor cell (11, 85, 87).

The results of treatment of primary F-prime donor cells with acridine orange (AO) indicates that the F-prime factor carries a gene(s) necessary for cell survival. Consequently viability during AO treatment depends upon integration of the F-prime factor into the host chromosome (11, 16, 85, 87). Since the chromosome contains a deletion of the F-prime segment, integration must take place at different regions on the bacterial chromosome by undergoing "non-allelic" pairing and recombination (48). This results in a class of aberrant donors which transfer the chromosome from new origins and possibly in the opposite direction (11, 85, 87).

Secondary F-prime strains. When a primary F-prime factor is transferred to a F^- strain, partial diploidy results. These F-prime strains can mobilize the chromosome with the polarity of the parental Hfr and are called secondary F-prime donors (3, 11, 51). The early papers describing F-prime factors (3, 51) actually were describing secondary F-prime factors. Approximately 10% of the secondary F-prime donors transfer the chromosome while the other cells continue to transfer only the F-prime factor (3).

Sex factor affinity locus (sfa). Adelberg and Burns (3) proposed that the infectious F^+ factor has a low affinity for the chromosome and no preferential site of attachment. Following the rare event of Hfr formation, an F-prime factor is formed resulting in a primary F-prime strain (3). Following curing with AO the cell giving rise to this primary F-prime factor can be reinfected with F^+ or the primary F-prime factor and these new donors are capable of relatively high frequency of oriented chromosome-transfer (3). This is due to the recognition of the site on the chromosome at which the F^+ factor had originally integrated. They (3) inferred that this "sex factor affinity" (*sfa*) locus results by a reciprocal exchange during F-prime formation. Broda (15) suggested that there exists specific regions on the chromosome at which F^+ integration occurs to form Hfrs. Now, it is known that there are *E. coli* Hfrs with origins all around the chromosome indicating a random distribution of these sites for integration (19, 92). There are Hfr strains of *S. typhimurium* and *Salmonella abony* with points of origin in at least 17 different regions (83). The distribution appears to be random over the 45-138 min region of the 138 min map but there is no report of *Salmonella* Hfrs with an origin in the 0 to 45 min region. There is an abundance of Hfr strains of *E. coli* in this region.

There is no explanation for the numerous sites of chromosomal homology with F^+ that allows the rare occurrence of Hfr formation which arise by chance attachment of the F^+ factor to the host chromosome (3).

Aberrant donor strains derived from F-prime strains. Transposition and inversion Hfr strains resulting from primary F-prime factor re-integration into the host chromosome are known (11). The transposition Hfrs result from re-integration in a different site. The direction of transfer can either be the same as the parental Hfr or in the opposite direction (11). Inversion Hfrs result from the inversion of the F-prime factors which re-integrate into their normal site and transfer the chromosome in the opposite direction (11).

A secondary F-prime donor can be constructed from mating a primary F-prime derivative of an inversion Hfr and a non-inverted isogenic F^- strain of the inversion Hfr. Therefore, a crossover between a non-inverted segment on the F-prime factor and the homologous segment on the chromosome gives mobilization in one direction, and a crossover between an inverted segment on the F-prime factor and the non-inverted homologous segment on the chromosome gives mobilization in the other direction (11).

The sites of reciprocal exchange in transposition Hfr formation and the polarity of exchange is definitely nonrandom (11). These sites of pairing are the result of mutual recognition between regions of fortuitously similar nucleotide sequences. The probability of pairing is a function of the extent of the similar sequences. The relative orientation of the nucleotide sequences involved is the determining factor for the direction of polarity.

Chromosome-transfer mediated by F-prime factors. Chromosomal transfer mediated by F-prime factors requires a region of homology for synaptic pairing (76, 86). A reciprocal crossover occurs within this region of pairing. The denser single strand of sex factor DNA (94) breaks between its origin and terminus (86, 76, 94). The origin with the free 5' nucleotide is the lead end in chromosome-transfer (82, 69, 94). The terminus is the most distal segment transferred (3, 11).

After specific pair formation (76, 77), there is a delay of 8 to 10 min for initiation of transfer of chromosomal markers in F-prime strains compared to their analogous Hfr strains. The rate of chromosome-transfer is the same for both F-prime and Hfr strains.

There are also secondary F-prime male strains known as Type I or Type II (76). Type I donors give 3 to 10 times as much F-prime transfer as chromosome-transfer, but Type II donors give higher frequencies of recombination for chromosomal markers and proximal F-prime markers. Type I donors change to Type II donors after storage at 5 C for several weeks followed by subculture in minimal medium (76). Type II donor strains must have a higher frequency of crossing over between the F-prime factor and chromosome.

The F-prime donors of *S. typhimurium* carrying at least the *trp* operon are similar to Type II males (84).

Independent transfer of both chromosome and F-prime factor occurs either extremely rarely or not at all (84), unlike the high frequency of independent transfer in *E. coli* (86).

Part III

Donor Strains Harboring More Than One Sex Factor (F)

Clark (22) isolated a double male strain of *E. coli* K12 by crossing two Hfr strains. The resulting recombinant is a haploid monokaryotic Hfr containing two chromosomally integrated sex factors. This strain is viable, stable, and transfers its genetic material to recipients in the form of two non-homologous linkage groups. Any given cell appears to transfer one or the other linkage group, but not both.

Echols (34) reported that an F-prime strain harboring F*gal*$^+$ excludes or destroys a superinfecting F*lac*$^+$ episome. Maas (64) isolated an Hfr recipient that completely excludes a superinfecting F*lac*$^+$ episome.

Bastarrachea and Clark (8) experimentally synthesized a strain of *E. coli* K12 harboring three sex factors. The donor is an F-30 merodiploid and the recipient is an F$^-$ phenocopy of the strain harboring two integrated sex factors (22). Chromosome-transfer is detected from both origins due to the two integrated F factors in addition to the autonomous transfer of the F-30 merogenote. F-30 is lost spontaneously.

Maas and Goldschmidt (65) isolated a recombination deficient (recA) Hfr strain containing a mutation most likely in the integrated F factor which permits the co-replication of an integrated and a free F factor. The F factor is F'lac. They did not find a wild type strain harboring two free F factors or one free and one integrated. Palchoudhury and Iyer (75) found a chromosomal mutation (DNA-t^s43) that leads to termination of DNA synthesis at 42 C which permits the cohabitation of two F-prime factors at the permissive (31 C) temperature. This lack of entry exclusion and intracellular incompatibility of one F-prime factor for another may result from an alteration in the membrane for the membrane-replication complex which is unstable at 42 C.

Joset et al. (56) isolated an Hfr strain following ultraviolet (UV) treatment of an Hfr strain that transfers the chromosome in the opposite direction and from a new origin. The Ra-1 Hfr strain also gives rise to RaF^+ (F^+) cells spontaneously (61). Further experiments by Low (61) indicate that the Ra-1 Hfr culture actually gives rise to the cells transferring from the second site (Ra-2 Hfr) by a detachment of the F from its Ra-1 site of integration and reassociation at the Ra-2 origin. Certain Ra-2 Hfr cells can transfer the chromosome like the Ra-1 Hfr. The mating properties of the normal F^+ in cured RaF^+ cells, RaF^+ in a normal F^- cells and an F^+ revertant from the Hayes Hfr in cured RaF^+ and normal F^- cells are those of the strains now harboring them. Low (61) concluded that the E. coli K12 RaF^+ strain (including Ra-1 and Ra-2) carries a normal sex factor but possesses chromosomal irregularities which give rise to the mating behavior characteristic of the Ra system in which there is a preference of RaF^+ to integrate into one of two specific chromosomal loci and only rarely in other sites around the chromosome. There apparently exists a segment of chromosomal DNA having sufficient homology with the F factor to allow reciprocal crossover and integration.

Kahn (57) presented an elaborate scheme for the evolution of a chromosomal locus responsible for two directional chromosomal transfer from one origin based upon a tandem duplication of Col V in the host chromosome.

Devries and Maas (33) described the isolation of double male strains in E. coli by mating various F-prime donor strains and a recA$^-$ Hfr recipient and selecting for recombinants which can act as early donors of both markers. These recombinants may be mutants in which the incompatibility barrier has been lost or which have two integrated sex factors. Further analysis indicates that all of the selected recombinants are indeed double Hfr strains. The

F-prime factor integrates into a region of the chromosome homologous to the chromosomal genes carried by the F-prime factor. Insertion is in the same direction as the F-prime mediated chromosomal transfer of the parental strain. An exception is the observation of a strain which transfers the chromosome in the opposite direction. This means that the orientation of chromosomal genes on the episome is opposite to that of the corresponding genes on the chromosome. It makes it unlikely that a reciprocal crossover takes place between homologous genes of chromosomal origin. The insertion process has a high degree of specificity which is evident by the constancy of the resulting double Hfr strain, even in the absence of an intact bacterial recombination system (33).

Part IV

Stages of Bacterial Conjugation

Specific pair formation. De Haan and Gross (31) defined specific pair formation as a donor-recipient cell union that is stable during gentle dilution. Curtiss *et al*. (27) published electron micrographs of presumed specific pair formation. The F^+, F-prime and Hfr donor cells possess F-pili (14) which react with specific f^+ antiserum and are the sites of attachment of F donor-specific phage. It is generally believed that the presence of donor pili is essential for specific pair formation (13, 14, 27, 93). It can be seen in electron micrographs that donor-specific RNA phage which attach to the sides of the sex-pilus outline the F-pili that appear to be making contact with the recipient cell (27, 93). The removal of F-pili by blending results in the temporary loss of ability for the donor to form specific pairs but is regained upon resynthesis of the F-pili (13, 27).

Curtiss et al. (27) suggested that donor cultures which are grown anaerobically prior to mating have a higher mean number of F-pili per cell, longer F-pili, a higher probability of forming specific pairs with F^- cells and a faster rate of initiation of chromosome-transfer than cells grown aerobically. A rich medium is superior to a completely synthetic medium (27). During periods of starvation, amino acid auxotrophic donor cells lose their F-pili, the ability to adsorb donor-specific phage, the ability to form specific pairs with F^- cells and they become more recipient-like (27). Certain transfer-defective mutations affect donor pili formation; these mutants cannot form specific pairs or transfer genetic material to recipients (1, 70, 71). Therefore, it appears that the F-pili act like grappling hooks and are necessary for specific pair formation with recipient cells (7). Normal donors do not mate with DNA-deficient minicells isolated from F^+ or F-prime minicell producing strains (24). The fact that a class of donor pili-less mutants have recipient abilities like the donor from which they arise (70) implies that the mere presence of F-pili is not sufficient to prevent donor:donor specific pair formation (26). From studying another class of donor pililess mutants which have recipient ability like a normal recipient strain, Ohtsubo (70) localized single mutations of the F-factor which apparently affect the synthesis of a regulatory product that permits the synthesis of both donor pili and some other product necessary for preventing donor:donor matings.

Another explanation for why donor cells form specific pairs with recipient cells may be that the donor cell possesses a cell surface structure that is responsible for donor exclusion which is nonantigenic or associated with the cell membrane (26). The only known antigenic structures that differ between donors and recipients are the f^+ (donor pili) and i^+ (somatic pili) antigens in donors (26).

Specific pair formation can occur in the absence of all energy metabolism on the part of either or both parents (30).

Effective pair formation. Effective pair formation is defined as the process by which specific pairs establish cellular connection through which genetic material can be transferred (26). The nature of the conjugation bridge has not been established unequivocally. The direct relationship of donor ability and the presence of

F-pili (13, 14, 27, 28, 93) indicates that the F-pili play some vital role either as the conjugal bridge or in the formation of the conjugal bridge.

Brinton *et al*. (14) suggested that the F-pili are very similar to non-sex specific I-pili with respect to gross physical structure. The F-pili have an axial hole of 2.0-2.5 mu in diameter, running the length of the pilus, thus providing the space for the passage of DNA through the pilus. During conjugation there does not appear to be significant transfer of any material other than DNA (44). However DNA has not yet been isolated in F-pili. Rosner *et al*. (81) found no detectable transfer of β-galactosidase during matings between F^+ and F^- cells. Silver (89) and Silver *et al* (90) found essentially no RNA or protein transferred during conjugation.

Ohtsubo (70) isolated donor-defective mutants possessing F-pili that are able to form specific pairs. This argues for genetic functions of the F factor that may be necessary for effective pair formation and/or for chromosome or F-factor transfer (26).

Curtiss (26) proposed a model for effective pair formation based on available data and some hunches as follows. After specific pair formation involving an interaction between the recipient cell surface and the tip of a donor pilus, the pilus is withdrawn into the donor cell, with the expenditure of energy, so as to achieve wall-to-wall contact between donor and recipient cells. Formation of a conjugation tube can then occur either by use of a component of the donor cell wall or membrane, or possibly by the hole in the donor cell surface remaining after withdrawal of the pilus.

Chromosome and conjugal fertility mobilization. This step prepares the circular donor chromosome and/or sex factor for linear sequential transfer. This process may occur during specific and effective pair formation or after effective pair formation (26, 27).

Jacob and Brenner (52) and Jacob *et al*. (53) proposed that chromosomal mobilization is initiated in the donor parent upon receiving a contact stimulus from the F^- parent. They proposed that chromosome-mobilization is related to vegetative chromosome replication which can be controlled by two chromosomal loci. One locus specifies the synthesis of an initiator probably a protein (53), and the other a replicator that recognizes the initiator and controls the direction of sequential chromosome-replication. They apply this two loci replication control model to autonomous F factors as well and suggest the simultaneous loss of these functions when F integrates into the chromosome. In their model they propose that the contact stimulus received from the recipient parent triggers the synthesis of the F-specified initiator, which acts to cleave the circular chromosome at F allowing for the linear sequential transfer of the DNA (52, 53).

However their model (52, 53) is based on the transfer of double stranded DNA from the donor to the recipient.

The rolling circle model for DNA replication of Gilbert and Dressler (39) suggests the transfer of a single strand of DNA resulting from a cleaving of a single strand at the site of F integration. The sex factor can be inserted into the *E. coli* chromosome with the origin facing either direction (69, 82). Therefore, depending on the orientation of the inserted sex factor, either strand of the *E. coli* chromosome can be attached to a particular strand of F.

Chromosomal or conjugal fertility factor transfer. Chromosomal and conjugal fertility factor transfer is the process of transferring the genetic material from the donor to the recipient cell. Currently it is believed that only a single strand of DNA is transferred during conjugation (12, 24, 39, 44, 69, 82, 94). The rolling circle model for chromosome replication of Gilbert and Dressler (39) is an ideal explanation for chromosomal transfer during conjugation. They postulate that replication begins by nicking one strand of the chromosome at a specific point. This may be at the origin of the autonomous or integrated sex factor too. Then the open strand with the exposed 5' terminus attaches to a cell membrane site for replication or to a site at the conjugal bridge for transfer. As this strand (positive) is peeled off and transferred, it is replicated in the recipient. The nontransferred strand (negative) remains closed. The positive strand is transferred to the recipient as a template and is replicated as short pieces by 3' to 5' growth of DNA and joined by the ligase. The negative strand receives its complementary strand simultaneously during the peeling away of the old complementary strand in the normal manner.

Curtiss _et al_. (26, 28) described experiments utilizing recombinant production and zygotic induction of prophage from different combinations of donor and recipient strains which can or cannot ferment the available carbohydrate source to determine that chromosome-transfer depends upon active metabolism in the donor to initiate chromosome-transfer and active metabolism in the recipient to control the rate of chromosome-transfer.

The conclusions of Bonhoeffer and Vielmetter (12) that chromosomal transfer is independent of DNA synthesis in the Hfr parent and dependent on DNA synthesis in the F^- parent is at odds with other published data and conclusions on the role of DNA synthesis during bacterial conjugation. From the use of DNAts mutations in F^+, F', Hfr donors and minicell (6) recipients, it is known that the amount of DNA synthesized in the donor parent is equal to the amount of DNA transferred to the minicells (R. Curtiss, R. L. Seigel, D. R. Stallions, and G. Van

Denbos, Bacteriol. Proc., p. 35, 1970). Therefore, DNA transfer during conjugation is accompanied by DNA synthesis in the Hfr parent and is not dependent on DNA synthesis in the F^- parent. They believe that this DNA synthesis in the donor during transfer is under separate control from vegetative chromosome replication. Stallions and Curtiss (91), by using DNAts mutants, concluded from a reinvestigation of the experiment of Bonhoeffer and Vielmetter (12) that chromosome-transfer from donors to recipients unable to replicate DNA at 42.5 C during vegetative growth occurs at normal frequencies when the mating is conducted at 42.5 C. Therefore some stage in haploid recombination formation is adversely affected in DNAts recipients mated at the temperature restrictive for DNA synthesis (91).

Marinus and Adelberg (66) studied different DNAts mutations located in at least two different genes on the chromosome with one of the 8 mutations present in each of 8 mating pairs. They (66) demonstrated that genetic transfer occurs normally in DNAts F^- strains mated at 42.5 C. Therefore, DNA synthesis in the F^- parent is not required for genetic transfer. They concluded that vegetative replication of the chromosome and transfer replication of F are separate processes with the former requiring at least two gene products which are nonessential for the latter (66).

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Curtiss et al. (28) and Cohen et al. (24) showed that DNA synthesis in the Hfr donor can force transfer of several percent of the donor chromosome to the recipient. Also F and short F-prime factors can be transferred. However, effective homologous pairing between the recipient chromosome and episome is necessary for transfer of longer F-prime factors and for chromosome-transfer mediated by F and F-prime factors (28). The F^- parent winds in the donor chromosome with the expenditure of energy (28). This process ensures pairing of homologous regions of the donor and recipient for recombination.

Recombinant formation. Recombinant formation requires synaptic pairing of homologous regions of the donor and recipient chromosomes following chromosome-transfer to enable crossovers to take place which are necessary for the integration of transferred markers. This is followed by reassortment of the donor and recipient genetic information which yields new combinations of genetic information. Finally there is segregation of recombinant chromosomes from nonrecombinant chromosomes.

Pittard and Walter (79) and Curtiss et al. (28) reported that the homologous pairing of donor and recipient chromosomes is necessary for the initiation of recombinant production.

The coinheritance of two donor markers in the same recipient depends upon the distance in transfer time between them. Linkage of less than 50% indicates a random coinheritance of the two markers (55). There is random coinheritance of proximally unselected markers that are more than 15 to 25 min of transfer time from the selected distally transferred marker. As the distance decreases, the frequency of coinheritance approaches 100%. Also, the coinheritance of distally transferred unselected markers with proximally transferred selected markers drops below 50% (or random linkage) when more than ten minutes of transfer time separates the two markers (55).

Pittard and Walker (79) and Glansdorff (40) concluded that genetic exchange almost always occurs near the origin of F-prime or Hfr chromosome transfer and the only significant exclusion in inheritance of donor markers occurs with markers less than 1 min of transfer time from the origin. Glansdorff (40) also found that two or more very proximally located markers may give the idea of being transposed when they are actually pseudotranspositions based on their kinetics of transfer.

Several models have been proposed to explain this low recovery of very early markers (26, 38, 40, 79). Walker and Pittard (97) reported that low recovery of very early donor markers in recombinants is not caused by the presence of sex-factor DNA at the leading end of donor DNA transferred during conjugation when using an isogenic Hfr phenocopy as the recipient. They found that recombination frequencies for a selected allele is as low as when a female strain is the recipient. These results do not rule out the possibility that a piece of sex factor DNA forms the lead end or origin of the DNA transferred by conjugation.

None of the recombination defective (rec$^-$) mutants studied has an effect on chromosomal mobilization and transfer in Hfr donors, but in the recipient strain they are unable to perform the functions necessary for haploid recombination (7, 100).

The model of recombinant formation proposed by Curtiss (26) based on the available data rules out a copy-choice type of recombination event. He proposed that the double stranded recipient chromosome undergoes regional melting at sites of single strand breaks to separate the complementary strands. The single stranded donor DNA then interacts with the recipient chromosome at these sites of regional melting. The effective homologous pairing may occur then by insertion of a portion of the single stranded donor DNA in place of the like strand of the recipient chromosome. Breakage then occurs in the other strand to produce a segment of inserted single stranded donor DNA. Synthesis of the strand complementary to the integrated donor strand proceeds in a 3' to 5' direction along the template, and when it is completed covalent bonds form between the ends of the polynucleotide strands. The resulting structure contains regions composed of parental donor and recipient DNA synthesized prior to mating with the donor segment composed of one strand synthesized prior to mating and one strand synthesized during recombination. The majority of the recombinant chromosome is the double stranded recipient DNA synthesized prior to mating.

Part V

Extrachromosomal DNA

Novick (68) defines "extrachromosomal element" as any hereditary unit that is physically separate from the chromosome of the cell and an independent replicon. Classifying an extrachromosomal element as either an episome or plasmid has met with considerable controversy recently (49, 68). Hayes (49) prefers to classify extrachromosomal elements as transmissible and nontransmissible plasmids. Transmissible plasmids include those extrachromosomal elements which can transfer themselves via conjugation and those which can transfer genetic units not linked to themselves. Nontransmissible plasmids cannot bring about their own transfer but can be transferred in association with a sex factor.

Many of the different transmissible plasmids have been isolated and characterized. These plasmids have all been isolated as covalently closed double stranded DNA molecules. To facilitate direct isolation of the plasmid from its natural host, Bazaral and Helinski (10) adapted the procedure of Radloff et al. (80) employing ethidium bromide (EtBr) in a preparative CsCl density gradient. Radloff et al. (80) used the dye EtBr, which intercalates between the base pairs of a double stranded DNA molecule causes a 12 degree unwinding of the helical structure. Waring (98) reported that the maximum amount of ethidium bromide that can bind to unwinding double stranded DNA is one molecule per every four or five base pairs. The supercoiled covalently closed plasmid molecules bind much less EtBr at saturating concentrations. Therefore, the unwinding double stranded DNA, both open circular and linear forms, bind much more dye resulting in a decrease in buoyant density. When the DNA-dye complexes are centrifuged to equilibrium in a CsCl density gradient, the supercoiled covalently closed plasmid molecules will band lower in the tube at a greater density than the open circular and linear forms.

Neutral sucrose gradients are used to determine the sedimentation coefficients of supercoiled DNA molecules (17). When these supercoiled molecules are centrifuged at pH values greater than 12, the molecules sediment at a faster rate. This is due to the more compact structure of the denatured supercoiled molecules (95).

Bazaral and Helinski (10) have determined that ColE1 supercoiled DNA has a molecular weight of 4.6×10^6 and a sedimentation coefficient of 23s in neutral sucrose. Olsen and Schoenhard (73) showed that the PO-1 and PO-2 plasmids of *Salmonella pullorum* MS53 have molecular weight of 1.5×10^6 and 45×10^6 daltons respectively and sedimentation coefficient in neutral sucrose of 17s and 65s respectively.

MATERIALS AND METHODS

Bacteria. *Salmonella pullorum* strain MS35, designated wild type, was selected from the stock collection of Dr. D. E. Schoenhard as the prototype organism from which auxotrophic recipient strains were derived (Table 1). The donor strains used for this investigation are listed in Table 2. *S. pullorum* strain MS53 was used as an indicator strain for the zygotic induction experiment and testing lysogenic derivatives of MS35. *Escherichia coli* AB312 was used for propagation of MS2.

The genotypic and phenotypic symbols suggested by Demerec *et al.* (32) were used.

The partial linkage maps of *S. pullorum* and *S. typhimurium* depicted in Fig. 1 and Fig. 2 respectively, show the relative position of the relevant markers and the point of origin and direction of transfer of the donor strains referred to in this investigation.

Bacteriophage. The temperate phage P35 described by Olsen (72) was induced from *S. pullorum* MS35 by zygotic induction. MS2 was the donor-specific RNA bacteriophage.

Phage were propagated and titered by a modification of a procedure described by Adams (2). Log phase bacterial

Table 1. Characteristics of *Salmonellae* recipient strains.[a]

Strain no.	Relevant genetic markers	Origin or ref.
MS35	*cys*-1 *cys*J1 *leu*-1	D. E. Schoenhard
MS369	*str*-1 *his*-1 *pro*-1 *thr*-1 *ilv*-1 *gal*-1	MS367
MS371	*str*-1 *his*-1 *thr*-1 *ilv*-1	MS367
MS374	*str*-1 *pro*-1 *thr*-1 *ilv*-1 *gal*-1	MS369
MS390	*str*-1 *thr*-1 *ilv*-1 *cys*E1	MS830 x MS374
MS391	*str*-1 *pro*-1 *ilv*-1 *cys*E1	MS830 x MS374
MS392	*str*-1 *pro*-1 *thr*-1 *cys*E1	MS830 x MS374
MS81	*leu*-1 *cys*E1	MS18
MS83	*leu*-1 *cys*E1 *pyr*D1	MS81
MS87	*leu*-1 *cys*E1 *pyr*D1 *arg*D1	MS83 (NTG)
MS88	*leu*-1 *cys*E1 *pyr*D1 *his*-5	MS83 (NTG)
MS90	*leu*-1 *cys*E1 *ilv*-3 *str*-1	MS81
MS92	*leu*-1 *cys*E1 *ilv*-3 *thr*-3	MS90
MS104	*leu*-1 *cys*B1 *trp*-3 *his*-3	MS103
SA1466	*pyr*B51 *met*-266 *ara*-9	K. E. Sanderson

[a]MS = *Salmonella pullorum*; SA = *Salmonella typhimurium*

Table 2. Characteristics of *Salmonellae* F-prime strains.[a]

Strain no.	Relevant genetic markers	Origin or ref.
MS810	*cys*-1 *cysJ*1 *leu*-1 *his*-4 *trp*-2/F-*trp*$^{+}$ (F71)	MS807
MS8300	*leu*-1 *cysE*1 *pyrD*1/F-*cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (F77)	SA532 x MS83
MS830	*leu*-1 *cysE*1 *pyrD*1/F-*cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (F77)	MS8300
MS831	*leu*-1 *cysE*1 *pyrD*1/F-*cysE*$^{-}$ *rfa*$^{?}$ *pyrE*$^{?}$ (F77*cysE*$^{-}$)	MS8300 (Spontaneous mutation)
MS832	*leu*-1 *cysE*1 *pyrD*1/F-*cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (F77)	MS830 (Poisson distribution experiment)
MS901	*leu*-1 *cysE*1 *ilv*-3 *str*-1/F-*cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (F77)	MS830 x MS90
SA532	*met*-483 *cysE*396/F-*cysE*$^{+}$ *rfa*$^{-}$ *pyrE*$^{+}$ (F77)	K. Sanderson

[a]MS = *Salmonella pullorum*; SA = *Salmonella typhimurium*

Figure 1. Partial linkage map of the _S. pullorum_ chromosome showing the relative position of the genetic markers. The F-prime factors are indicated in the expanded portion.

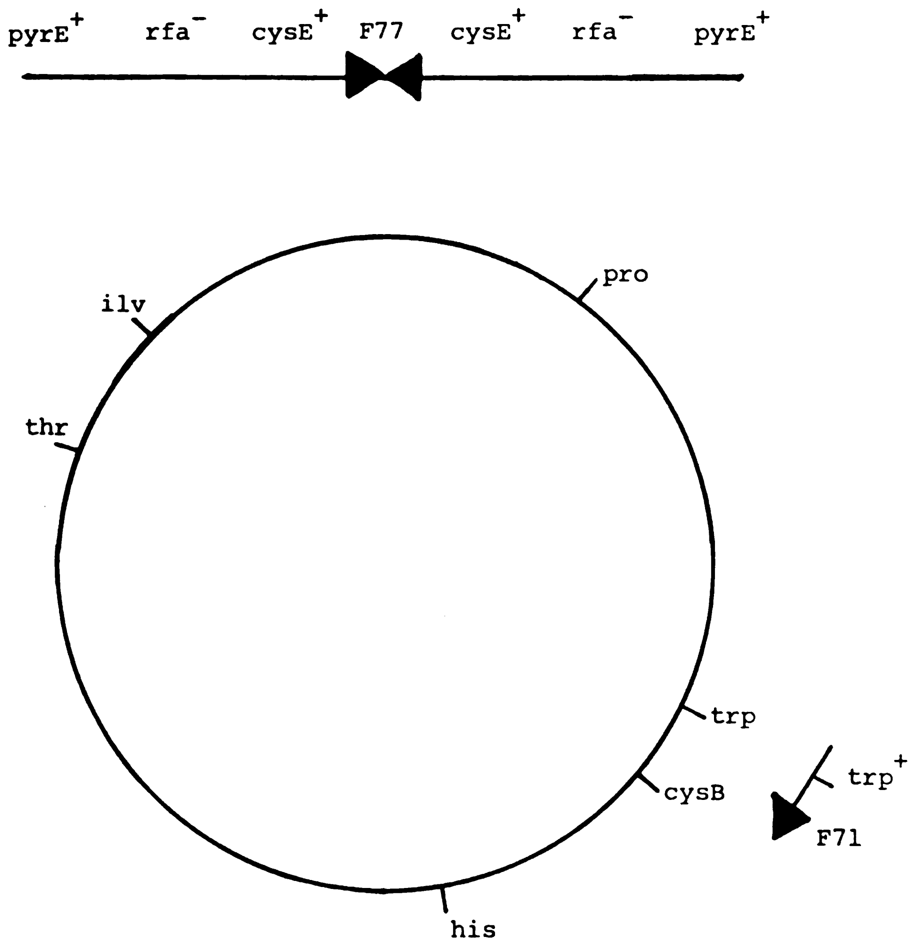
pyrE+
rfa−
cysE+
F77
cysE+
rfa−
pyrE+
pro
ilv
thr
trp
trp+
cysB
F71
his

Figure 1

Figure 2. Partial linkage map of the *S. typhimurium* chromosome showing the relative position of the genetic markers. The F-prime factors are indicated in the expanded portion.

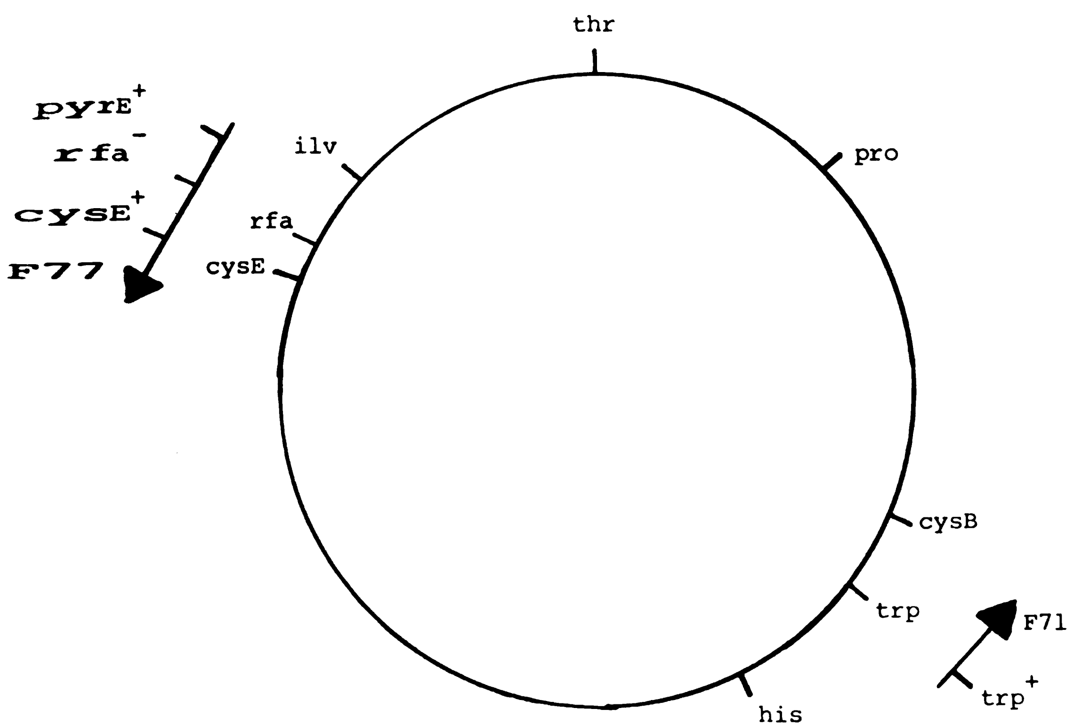

Figure 2

cells in aerated L broth were infected with phage at a multiplicity of infection (m.o.i.) of 0.1. The infected cells were incubated 18 hr with aeration by shaking at 37 C. One ml of chloroform was added to the culture, followed by vortexing the culture for one-half min. The chloroform treated cells were then reincubated at 37 C with aeration by shaking for 30 min. The bacterial debris was then removed by centrifugation for 10 min at 8000 x g. The phage in the supernatant fluid were stored over chloroform at 4 C. The phage were titered by assay of the number of plaque forming units (pfu) per ml by the soft agar overlay technique (2).

Media. The E minimal medium described by Vogel and Bonner (96) was supplemented with L-amino acids at a final concentration of 20 ug/ml and D-glucose (Pfanstiehl) at 0.4% (w/v) for the growth of amino acid auxotrophs.

L broth and L agar (1.5% Difco agar) containing 10 g of tryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl per liter of deionized distilled water were employed for routine cultivation.

When used, dihydrostreptomycin sulfate was added to a final concentration of 1200 ug/ml in minimal media.

Bacto SIM medium (Difco) was used to detect sulfide and/or indole production.

For radioactive labeling, the bacteria were grown overnight in TCGU broth containing 0.1 M tris (hydroxymethyl)aminomethane(Tris)-hydrochloride pH 7.4, 0.4%

vitamin-free casamino acids, 25 ug/ml of deoxyadenosine, 7 ug/ml uridine, and 0.4% glucose which was autoclaved separately and added prior to use. When labeling with ^{14}C-thymidine, 25 ug/ml of deoxyguanosine were added in addition to the TCGU broth.

Bacterial cultures were checked for specific antigens with antisera (Difco, Salmonella O antiserum group D factor 9 for *S. pullorum* and Difco, *Salmonella* H antiserum i for *S. typhimurium*).

Chemicals. The general chemicals used were reagent grade. Special chemicals are listed in Table 3.

Buffers and dialysis. TM buffer (Tris-maleic) was made in deionized, distilled water which contained: 0.05 M tris (hydroxymethyl)aminomethane(Tris)-hydrochloride and 0.05 M maleic acid, pH 6.0. The general buffer TES was made in deionized, distilled water which contained: 0.05 M tris (hydroxymethyl)aminomethane(Tris)-hydrochloride, 0.005 M (ethylenedinitrilo)tetraacetic acid (EDTA), and 0.05 M NaCl, pH 8.0.

Dialysis was performed using sterile dialysis tubing which had been boiled in 0.5 M EDTA pH 7.0 for 10 min, and then autoclaved in 0.05 M Tris, pH 8.0.

Mutagenic treatment. The uridine mutation was induced by N-methyl-N'nitro-N-nitrosoguanidine (NTG) following the method recommended by Adelberg, Mandel and Chen (4). Five ml of logarithmic phase cells (2×10^8 cells/ml) growing in E minimal broth were collected on a

Table 3. Chemicals and sources.

Chemicals	Source
N-methyl-N'-nitro-N-nitrosoguanidine (NTG)	Aldrich Chemical Company Milwaukee, Wisconsin
Ethidium bromide (EtBr)	Calbiochem Los Angeles, Calif.
Lysozyme (crystallized egg white)	Armour Pharmaceutical Co. Kankakee, Illinois
Cesium chloride (CsCl)	Schwarz-Mann Orangeburg, New York
Bovine albumin fraction V (BSA)	Pentex Incorporated Kankakee, Illinois
Brij 58	Emulsion Engineering Co. Elk Grove, Illinois
Antisera	Difco Laboratories Detroit, Michigan
2,5(diphenyloxazole)-benzene (PPO) 1,4,-bis 2(4-methyl-5-phenyloxazole)-benzene (POPOP)	Packard Instrument Co. Downers Grove, Illinois

millipore filter and resuspended in 10 ml of TM buffer pH 6.0 containing 100 ug of NTG/ml. The suspension was incubated at 37 C for 20 minutes with aeration. A 1 ml aliquot portion was filtered to remove the excess NTG, and then resuspended in 10 ml of E minimal broth supplemented to permit the growth of uridine mutants. The suspension was incubated with aeration for five generations.

Enrichment for the desired mutant was by the penicillin treatment described by Gorini and Kaufman (43). Ten ml of the NTG treated suspension (5×10^8 cells/ml) were centrifuged and the pellet resuspended in 1 ml of E minimal broth. A 0.1 ml aliquot portion of the resuspended pellet was added to 10 ml of E minimal broth supplemented with 10% sucrose, 0.5% glucose, and 0.01 M $MgSO_4$ and the growth requirements of the parental cell type. The culture was grown with aeration for 3 hr followed by the addition of 2000 units/ml of Penicillin G. Additional incubation was done at 37 C without aeration for 4 hr until approximately 50% of the cells had become spheroplasts. Then the action of penicillin was stopped by chilling the culture in an ice bath. The culture was centrifuged and the pellet resuspended in 10 ml of E minimal broth properly supplemented to permit growth of the uridine mutants. Following the second cycle of penicillin enrichment, the cells were plated on L agar plates and the uridine mutants isolated by replica plating to selective media. The uridine mutants were then replicated to selective media on which they were characterized (101) as shown in Table 4.

The histidine (*his*-5) and arginine (*arg*-1) mutations were induced in MS83 by NTG according to the method described by Glover (41). Five ml of logarithemic phase cells (2 x 10^8 cells/ml) growing in L broth were centrifuged and the pellet resuspended in 5 ml fresh L broth. NTG was added to a final concentration of 30 ug/ml and the suspension allowed to incubate at 37 C for 15 min with aeration. The cells were washed twice in E minimal broth and resuspended in 5 ml of E minimal broth. A 0.1 ml aliquot portion was added to 5 ml of L broth and incubated 37 C overnight with aeration to allow expression of the mutations. The cells were pelleted, washed in E minimal medium and resuspended in E minimal broth supplemented with the amino acid requirements of the parental strains and allowed to incubate at 37 C for 3 hr with shaking. Enrichment for the induced mutations was by the penicillin treatment of Gorini and Kaufman (43) as previously described. Following the completion of the penicillin enrichment treatment the cells were iced, pelleted by centrifugation in a Sorvall RC-2 centrifuge at 5 C and resuspended in 3 ml of E minimal broth. One-tenth ml aliquot portions of diluted resuspended cells were plated on L agar plates to permit the growth of approximately 200 colonies per plate.

Table 4. Growth requirements of pyrB, C, D, E and F mutants.

Locus	Carbamyl aspartic acid (CAA)	Dihydro-orotic acid (DHOA)	Orotic acid (OA)	Uracil or Uridine (U)
pyrB	+	+	++	+++
C	-	+	+++	+++
D	-	-	+++	+++
E	-	-	-	+++
F	-	-	-	+++

These isolated colonies were picked to fresh L agar plates and spread in patches to serve as master plates for replica plating to pools shown in Table 5 for the identification of the induced mutations.

The _arg_-1 mutation was further studied and identified as _argD1_. The mutant strain grows on citrulline but not on ornithine.

Presence of F factor. The method of Schleif (88) was used to test for the presence of the F-prime sex factor. The donor specific RNA bacteriophage MS2 was streaked down the center of an L agar plate and allowed to dry. The bacteria being tested were streaked across the bacteriophage. Bacteria harboring F showed a greatly reduced number at the intersection of the MS2 streak; F^- cells showed no reduction in number.

Isolation and characterization of donor strains of S. pullorum. The F77 factor was isolated from _S. typhimurium_ by Sanderson and carries the _cysE_$^+$ _rfa_$^-$ _pyrE_$^+$ genes. The origin and direction of chromosome-mobilization by F77 in _S. typhimurium_ are shown in Fig. 2.

The F77 factor was introduced into _S. pullorum_ by mating _S. typhimurium_ SA532 with MS83. Log phase cultures of the donor (1×10^8 cells) were mixed with the recipient (1×10^8 cells) and impinged upon a millipore membrane filter. The filter was removed to pre-warmed L agar plates at 37 C. The membranes were then inserted into

Table 5. Amino acid pools used for the determination of auxotrophic mutants.[a]

Pool number	1	2	3	4
5	phenylalanine	leucine	serine	glutamate
6	tryptophane	isoleucine	glycine	arginine
7	histidine	valine	cysteine uridine	proline
8	aspartic acid	methionine	threonine	lysine

[a]Each pool was supplemented with leucine and cysteine.

either 1 ml of E minimal broth with glucose and agitated in fluted test tubes on a Vortex Jr mixer to remove the mating pairs from the membrane and interrupt the mating, or placed into 1 ml of E minimal broth plus glucose in a 13 x 100 mm test tube and vibrated for 15 sec in an apparatus described by Low and Wood (63). The mating mixture was then diluted 1:3 in E minimal broth and 0.1 ml aliquot portions were dispensed into 3 ml L soft agar overlays and poured over the surface of supplemented E minimal agar plates to allow growth of only the desired recombinant type. After incubation at 37 C for 96 hr, the recombinants were re-streaked on the same kind of selective medium and reincubated at 37 C for 72 hr. Isolated colonies were then picked and inoculated into 3 ml of L broth and incubated at 37 C for 12 hr with aeration and then tested for sensitivity to MS2 phage. The tubes containing MS2 sensitive bacteria were then subcultured into SIM media to detect hydrogen sulfide, or indole production. The cultures appearing to be *S. pullorum* were then streaked for isolation on L agar and incubated at 37 C for 24 hr. Individual colonies were then tested for their response to Group D antisera and auxotrophic requirements. The donor MS8300 resulted from these manipulations. A partial characterization of *S. pullorum*, *S. typhimurium* and *E. coli* is shown in Table 6.

Table 6. Partial characterization of *S. pullorum*, *S. typhimurium* and *E. coli*.

Organism	Produces H_2S	Produces indole	Response to antiserum	
			Salmonella O group D factor 9	Salmonella H group i
S. pullorum	-	-	+	-
S. typhimurium	+	-	-	+
E. coli	-	+	-	-

A routine check of the parental auxotrophic requirements, MS2 sensitivity and donor fertility of MS8300, revealed an isolate with increased fertility, designated MS830, and an isolate with a cysteine requirement, designated MS831.

MS901 is a recombinant isolated from a mating of MS830 x MS90.

All donor strains constructed during this investigation were stable with respect to the F-prime factor when stored on L agar plates for periods up to 4 months at 4 C. L broth cultures were less stable when stored at 4 C for over 3 months. Therefore, spontaneous curing of the F-prime in S. pullorum donors was not a problem.

Techniques of bacterial mating. A modification of the millipore filter matings described by Godfrey (42) was employed for routine interrupted matings. The donor and recipient cells were grown overnight in L broth at 37 C with aeration. Following a 1:20 dilution into fresh L broth, the recipients were incubated at 37 C for 3 hr with aeration and the donors incubated at 37 C for 3 hr without aeration. The mating mixture contained a ratio of 1 donor to 10 recipients at a final concentration of 1×10^8 donors. The mating mixture was impinged upon pre-wet millipore HA 0.45 u, 25 mm sterile filters. The zero time was taken when the cells were drawn onto the millipore filter. The filter was placed immediately upon a moist prewarmed L agar plate and incubated for the desired period at 37 C. Following incubation, the millipore filter was removed from the agar surface to a 13 x 100 mm sterile test tube containing 1 ml of E minimal medium with glucose, and shaken for 15 sec with an apparatus described by Low and Wood (63) to separate conjugal pairs. Further dilutions were made in E minimal broth with glucose. One-tenth ml portions were pipetted from the mating mixture dilutions into tubes containing 3 ml of E minimal soft agar, 0.75%, kept at 45 C. The tubes were shaken and then the mixture was poured over the surface of E minimal agar plates selective for specific recombinants. The plates were incubated 96 hr at 37 C. Donor cells were counterselected by omitting an amino acid required of the donor from the E minimal agar plates.

Zygotic induction experiments were carried out exactly like millipore filter matings described above except that following interruption of mating pairs, the mating mixture was diluted into sterile physiological saline. One-tenth ml portions were transferred to melted L soft agar overlays containing 0.2 ml of logarithmic phase MS53 which served as the indicator strain. The L soft agar overlays were then poured over the surface of L agar plates. Following incubation at 37 C for 24 hr, the phage titer for each time interval was calculated.

To allow for the putative 40 min latent period of the phage growth cycle, the diluted mating mixture that was plated at each time interval was reincubated at 37 C for 40 min. One-tenth ml portions were then plated as described above.

Linkage analysis--scoring unselected markers. The selected recombinant colonies were purified by restreaking them on the same type of selective minimal agar medium. After incubation for 96 hr at 37 C, isolated colonies were picked and spread as patches onto the same type of selective minimal agar medium. Following 72 hr incubation at 37 C, these patch plates served as master plates for replica plating to various types of selective minimal media agar plates to determine linkage of the selected markers to unselected markers.

Kinetic analysis--time of entry of genetic markers. The kinetic studies were done to demonstrate that the gradients of transfer were due to F-prime mediated chromosomal transfer and not to random F^+ type of chromosomal transfer. The time of entry of the genetic markers was determined by interrupting millipore filter matings at 10 min intervals.

Cross streak method. This technique is based on a modification of the procedure of Berg and Curtiss (11). A loopfull of logarithmic phase donor cells of approximately 2 x 10^8 cells/ml was streaked across the dried line (0.02 ml) of the logarithmic phase recipient tester strain already applied to the surface of selective E minimal agar plates. Twelve donor cultures could be tested this way. The plates were incubated for 96 hr at 37 C. The recombinants were scored and the donor strains yielding the most recombinants were selected for use in further mating studies.

Poisson distribution for selecting fertile donor strains. A logarithmic phase culture of approximately 2×10^8 donor cells/ml was diluted to 10 cells/ml and 0.1 ml aliquot portions were added to 3 ml of L broth and incubated overnight at 37 C with aeration. When there was no growth in at least 37% of the broth tubes, there is an average of one cell per tube. A loopful of the donor cultures was tested by the cross streak method of bacterial mating. The appearance of recombinants was taken as evidence for the selection of a fertile donor strain arising from one cell.

Lysogenization of *S. pullorum* MS35 by P35. Logarithmic phase cultures of *S. pullorum* MS35 were infected with a high m.o.i. with phage P35 and incubated overnight at 37 C with aeration. A loopful of the overnight culture was streaked for colony isolation on an L agar plate and incubated at 37 C for 24 hr. Isolated colonies were subcultured into 3 ml of L broth and incubated at 37 C with aeration through early log phase. A loopful of each culture was spotted on a fresh lawn of MS53 indicator strain sensitive to P35 and incubated at 37 C for 8 hr. Lysis of MS53 occurred if the culture tested was lysogenic for

P35. Lysogenization had no effect on auxotrophic requirements or mating type.

Electron microscopy. Photographs of various S. pullorum donor and recipient strains were printed from developed Estar thick base plastic film (3¼ x 4 in) that had been exposed in a Philips EM300 electron microscope. Both collodion and formvar coated grids were used. The optimum stain was 0.5% phosphotungstic acid (PTA) at pH 7.5.

An overnight L broth culture of the bacteria to be examined was impinged upon a millipore HA 0.45 u, 25 mm membrane filter. The filter was placed in a 13 x 100 mm test tube containing 3 ml of sterile, distilled water. The test tube was gently shaken to wash the cells off of the filter. The cell suspension was diluted to about 2×10^8 cells/ml and a drop was placed on a grid for viewing.

To show the attachment of the RNA containing male-specific phage MS2 to the sex-pili of S. pullorum, the phage were added at a m.o.i. of 100 to the cells after they had been washed off the millipore membrane filter in a 13 x 100 mm test tube containing distilled water. The mixture was allowed to sit at room temperature for approximately 30 min before placing a drop of the sample on the grid for viewing.

To photograph mating pairs, 1×10^9 donors were mixed with 1×10^9 recipients in L broth and incubated

15 min at 37 C in a New Brunswick G76 gyrotory shaker at a speed setting of 3. The mating pairs were then impinged upon a millipore HA 0.45 u, 25 mm membrane filter and incubated for 15 min at 37 C on a prewarmed moist L agar plate. The mating pairs were washed from the filter into 1 ml of distilled water in a 13 x 100 mm test tube by gently swirling the test tube. MS2 phage were added at a m.o.i. of 100 and the mixture incubated 20 min at 37 C. The mixture was then stored in the cold until used (never more than 1 hr). A drop of the sample was placed on the grid for viewing.

Radioactive labeling and counting. The ^{3}H-thymidine (15 Ci/m mole) was purchased from Calbiochem, Los Angeles, Calif., and ^{14}C-thymidine (57 mCi/m mole) was purchased from Amersham/Searle, Chicago, Ill. The cells growing in TCGU broth containing deoxyadenosine were labeled with ^{3}H-thymidine at 1 uCi/ml of broth or ^{14}C-thymidine at 0.125 uCi/mi of broth supplemented with deoxyguanosine. To allow maximum incorporation of the ^{3}H-thymidine, S. pullorum cultures were incubated 12 hr at 37 C with aeration. S. typhimuirum cultures were incubated 6 hr at 37 C with aeration. The S. pullorum MS35 derivatives used in labeling experiments incorporated approximately 70% of the added ^{3}H-thymidine. The S. typhimurium strains incorporated 25% of the added ^{3}H-thymidine and ^{14}C-thymidine.

The scintillation mixture used for cell radioactive counting contained 1.35 gm of 2,5(diphenyloxazole)-benzene

(PPO) and 27 mg of 1,4,-bis 2(4-methyl-5-phenyloxazole)-benzene (POPOP) per liter of toluene. Ten ml of this mixture were added to vials containing the dried radioactive samples on filter paper and counted in a Packard Model 2002 Tri-Carb Liquid Scintillation Spectrometer.

Preparation of bacterial lysates. The lysate preparation procedure of Clewell and Helinski (23) was modified for isolation of plasmid DNA. Cells were grown in 30 ml of TCGU broth containing radioactive thymidine at 37 C with aeration. The labeled cells were pelleted by centrifugation in a Sorvall RC-2 at 10,000 rpm for 15 min at 5 C. The pellet was resuspended in 1 ml of cold 25% sucrose in 0.05 M Tris, pH 8.0, transferred to a polycarbonate test tube and plunged into an ice bath. Two-tenths ml of lysozyme (5 mg/ml in 0.25 M Tris, pH 8.0) were added and the mixture iced for 5 min, followed by the addition of 0.4 ml EDTA made 0.25 M at pH 8.0 and another 5 min on ice. The cells were then lysed by the addition of 1.6 ml of the "lytic mixture" containing 1% Brij 58, 0.4% sodium deoxycholate, 0.0625 M EDTA and 0.05 M Tris pH 8.0. The mixture was again iced for 15 min. Lysis was completed by transferring the mixture to Beckman cellulose nitrate test tubes and subjected to 5-7 cycles of freeze-thawing. Freezing was accomplished by plunging the cellulose nitrate tubes into an ethanol-dry ice bath followed by thawing in a 45 C water bath. Lysis was complete when the mixture turned from opaque to transparent, and increased in viscosity. This crude lysate was then transferred to a small plastic centrifuge tube and centrifuged in a Sorvall RC2-B at 20,000 rpm (48,000 x g) for 30 min at 5 C. The pellet contained approximately 95% of the chromosomal DNA leaving the plasmid DNA in the supernatant fluid. The supernatant fluid was now referred to as the cleared lysate.

Dye-buoyant density equilibrium centrifugation. A modified procedure of Bazaral and Helinski (10) was used to isolate plasmid DNA in a CsCl-EtBr solution. Three ml of the ^{3}H-thymidine or ^{14}C-thymidine labeled cleared lysate from a 30 ml TCGU supplemented culture were mixed with 2.7 ml of TES, 0.5 ml of ethidium bromide (5 mg/ml TES) and 6 gm of anhydrous CsCl (final density of 1.54 gm/ml). The mixture was poured into a polyallomer tube that had been pretreated by boiling 15 min in TES buffer and soaked in 100 ug BSA/ml TES for 1 hour. The mixture was then covered with a layer of sterile light mineral oil and the tube capped and centrifuged in a Type 50 rotor at 44,000 rpm for 30 hr at 15 C in the Beckman model L3-50 ultracentrifuge.

Approximately 60 fractions (12 drops each) of 0.1 ml were collected directly into autoclaved 12 x 75 mm polypropylene tubes by puncturing the bottom of the polyallomer gradient tube with a #24 gauge needle. Five ul samples of each fraction were spotted on 3/4" squares of

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Whatman #1 filter paper, washed in TCA, ethanol and anhydrous ether, dried and counted as described above.

The plasmid peak fractions were pooled and dialyzed overnight at 5 C in the dark in TES buffer to remove the EtBr and CsCl.

Sucrose density gradients. A 0.15 ml sample of *S. pullorum* or 0.3 ml sample of *S. typhimurium* cleared lysate or pooled, dialyzed fractions was layered directly onto a 5.2 ml linear 20-31% neutral sucrose gradients made in 0.005 M EDTA, 0.5 M NaCl and 0.05 M Tris, pH 8.0. Centrifugation was in a SW50L rotor at 50,000 rpm for 90 min at 15 C. Approximately 32 fractions of 0.17 ml each (8 drops) were collected from the bottom of the tube by using a Beckman fraction recovery system. The fractions were collected directly onto 3/4" squares of Whatman #1 filter paper, dried under a heat lamp, washed successively in 250 ml of cold 5% TCA, 95% ethanol and anhydrous ether. The filter paper fractions were then dried, and placed in vials containing 10 ml of toluene scintillation fluid and counted as described above. For cosedimentation experiments, two samples from the dialyzed pooled fractions in the amounts described above were layered on top of each other on the neutral sucrose gradients and centrifuged as described above for the single samples.

RESULTS

Part I

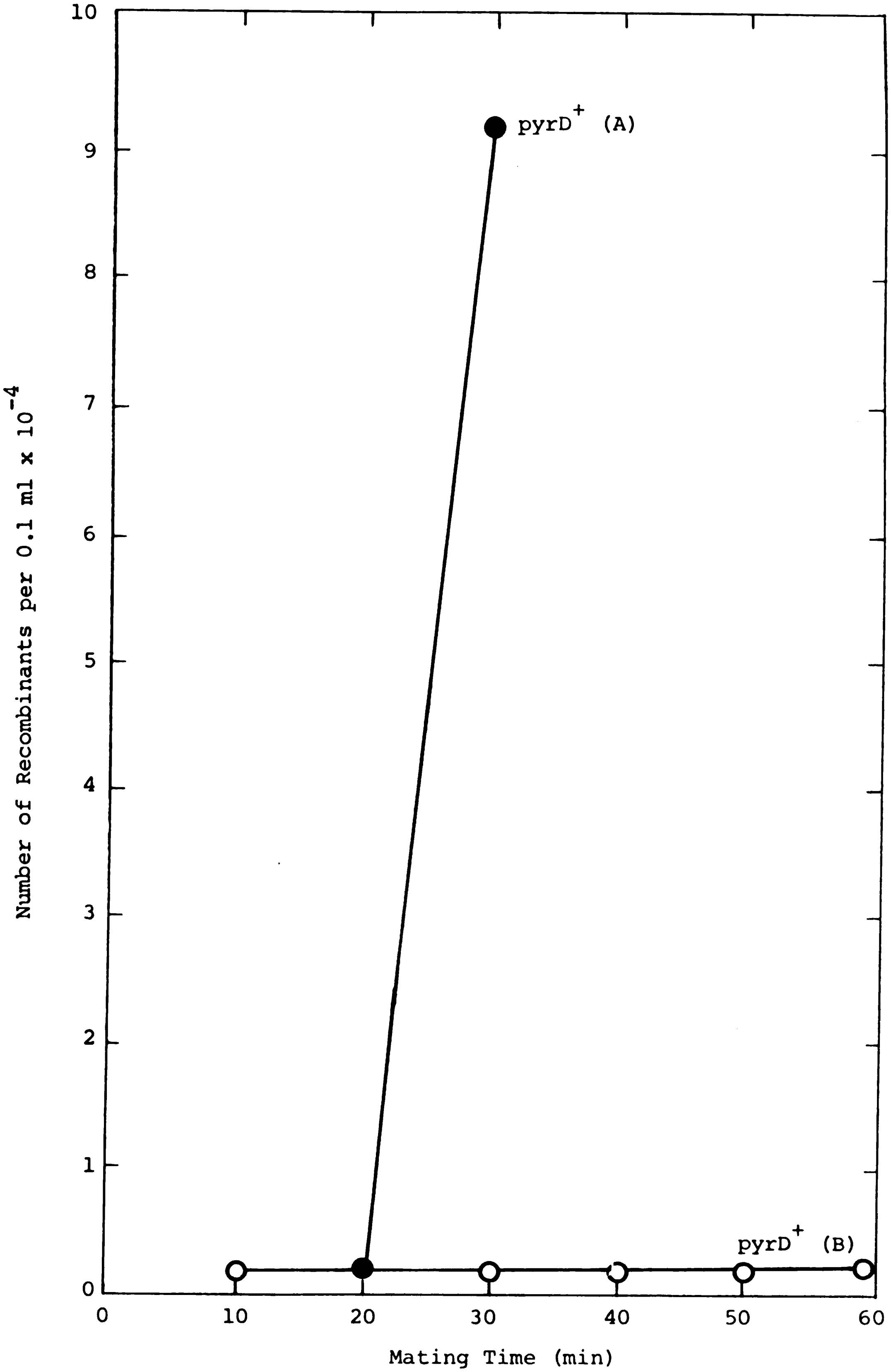
Counterselection of Donors Carrying F77

The F77 transmissible plasmid in *S. typhimurium* transfers the host chromosome in the clockwise order O-*cysE*-*ilv*-*thr*-*pro*. Godfrey (42) observed that F77 transfers the host chromosome of *S. pullorum* from one origin between *ilv* and *pro* in two directions (Fig. 1).

A very stable mutant of *S. pullorum* MS81, which harbors a *pyrD1* mutation, was selected and designated MS83. An isolate of MS83 infected with F77 was designated MS8300. A stable derivative of MS8300 which transferred the host chromosome at a higher frequency was designated MS830 and used for the mating experiments described in this thesis.

To map the *pyrD1* mutation, the donor MS810 carrying F71 and the recipient MS83 were mated and $PyrD^+$ recombinants were selected. The F71 transmissible plasmid carrying trp^+ mobilizes from *trp* in a counterclockwise direction in *S. pullorum* (42). The *pyrD* gene was mapped in *S. pullorum* (Fig. 3) in the same relative position it

Figure 3. Time of entry of the *pyrD*$^+$ gene from MS810 x MS83 (A) and MS901 x MS83 (B) matings. Matings took place on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (1×10^6 donor cells) was plated at each time interval on media selective for PyrD$^+$ recombinants. The selective media was supplemented with leucine and cysteine. Histidine (A) and isoleucine (B) auxotrophy were used for counterselection.



pyrD+ (A)
pyrD+ (B)
Number of Recombinants per 0.1 ml x 10-4
Mating Time (min)
0
1
2
3
4
5
6
7
8
9
10
0
10
20
30
40
50
60

Figure 3

mapped in *S. typhimurium*, which was approximately 10 minutes counterclockwise from the *trp* operon. To determine that *pyrD* was not located near the origin of transfer for F77 in *S. pullorum*, the MS901 x MS83 mating served as the control.

Part II

Origins of Transfer and Genetic Loci Mapped with Donors Carrying F77

Matings using the donor MS830. The recombination frequencies and gradients of transfer determined from matings between the donor MS830 and various *S. pullorum* recipients are listed in Table 7.

It appeared from the matings with MS830 x MS374 (Table 7) that F77 transfers the *S. pullorum* chromosome in the order O-*ilv*-*thr*-*pro*.

From the analysis of the linkage data (Table 8) and kinetic studies (Fig. 4) of the MS830 x MS374 matings, it was concluded that the linear arrangement and orientation of these selected genes in *S. pullorum* was O-*ilv*-*thr*-*pro*. The data on the incidence of coinheritance of the donor MS830 *cysE1* auxotrophic marker and streptomycin sensitivity gene are presented in Table 9. There was more coinheritance of the donors streptomycin sensitivity gene than the *cysE1* gene.

The results from the MS830 x MS369 matings (Table 7) were interpreted as a gradient of transfer continuous

Table 7. Recombination frequencies and gradients of marker transfer in crosses with S. pullorum donors and recipients.

Cross	Counter-selection	Length of mating (min)	Selected recombinant	Recombination frequency (per initial donor input)	Relative frequency
MS8300 x MS374	pyrD1	60	Ilv^+	3.9×10^{-5}	1.00
			Thr^+	7.5×10^{-6}	.22
			Pro^+	1.5×10^{-6}	.05
MS830 x MS374	pyrD1	60	Ilv^+	1.2×10^{-4}	1.00
			Thr^+	6.7×10^{-5}	.54
			Pro^+	1.8×10^{-5}	.15
MS830 x MS369	pyrD1	60	Ilv^+	1.3×10^{-4}	1.00
			Thr^+	7.0×10^{-5}	.54
			Pro^+	1.8×10^{-5}	.14
			His^+	0.7×10^{-5}	.05
MS830 x MS371	pyrD1	60	Ilv^+	8.8×10^{-5}	1.00
			Thr^+	7.4×10^{-5}	.84
			His^+	4.2×10^{-6}	.05
MS830 x MS90	pyrD1	60	$CysE^+$	6.5×10^{-4}	1.00
			Ilv^+	1.1×10^{-4}	.17
MS830 x MS92	pyrD1	60	$CysE^+$	6.8×10^{-4}	1.00
MS830 x MS390	pyrD1	60	$CysE^+$	6.0×10^{-5}	1.00
			Ilv^+	1.3×10^{-5}	.22
			Thr^+	1.2×10^{-5}	.20
MS830 x MS104	pyrD1	60	Trp^+	1.4×10^{-5}	1.00
			$CysB^+$	1.0×10^{-5}	.71
			His^+	$.7 \times 10^{-5}$	.50
MS831 x MS369	pyrD1	60	Ilv^+	4.8×10^{-6}	.80
			Thr^+	7.0×10^{-7}	.12
			Pro^+	6.0×10^{-6}	1.00
			His^+	1.5×10^{-6}	.25
MS831 x MS374	pyrD1	60	Ilv^+	6.3×10^{-6}	.93
			Thr^+	1.8×10^{-6}	.27
			Pro^+	6.8×10^{-6}	1.00
MS831 x MS104	pyrD1	60	Trp^+	2.0×10^{-5}	1.00
			$CysB^+$	1.5×10^{-5}	.75
			His^+	2.4×10^{-6}	.12
MS832 x MS374	pyrD1	60	Ilv^+	4.7×10^{-4}[a]	1.00
MS832 x MS104	pyrD1	60	His^+	4.2×10^{-6}[a]	1.00
MS832 x MS374	pyrD1	60	Ilv^+	1.7×10^{-4}	1.00
			Thr^+	8.8×10^{-6}	.52
			Pro^+	1.5×10^{-6}	.01
MS832 x MS104	pyrD1	60	Trp^+	6.3×10^{-6}	1.00
			$CysB^+$	5.1×10^{-6}	.81
			His^+	2.7×10^{-7}	.04

[a]Initial observation

Table 8. Analysis of inheritance of unselected donor markers in recombinants from the MS830 x MS374 crosses.[a]

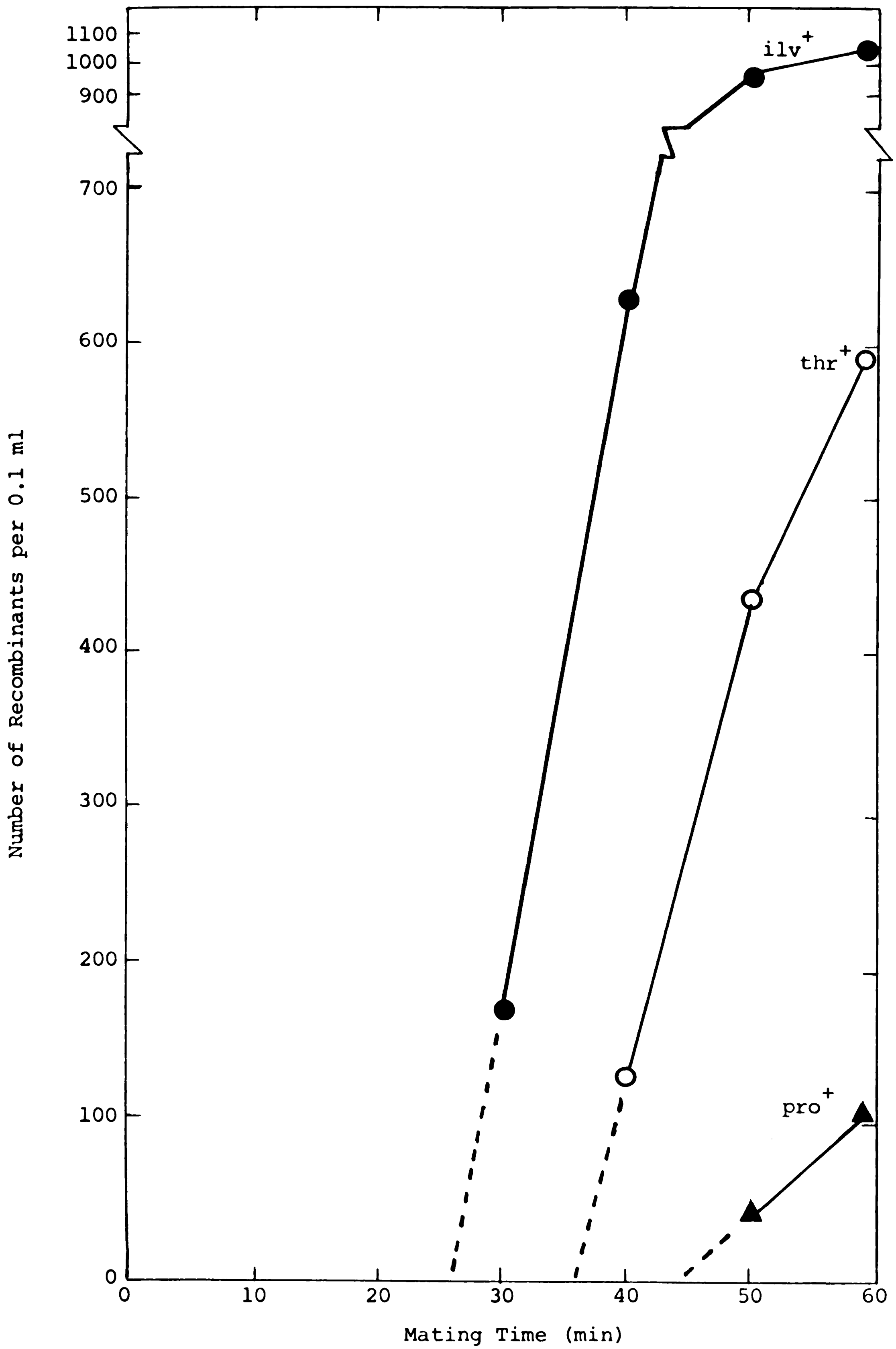
Unselected phenotype	Selected phenotype		
	918[b] Ilv^+	858 Thr^+	1189 Pro^+
Ilv^+	-	26.5	10.8
Thr^+	18.5[c]	-	16.5
Pro	8.1	15.2	-

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

Figure 4. Time of entry of various markers from MS830 x MS374 matings. MS830 was mated with MS374 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Ilv^+, Thr^+ and Pro^+ recombinants. The selective media were supplemented with leucine and cysteine. Uridine auxotrophy was used for counterselection.



Number of Recombinants per 0.1 ml
Mating Time (min)
ilv+
thr+
pro+
0
100
200
300
400
500
600
700
900
1000
1100
0
10
20
30
40
50
60

Figure 4

Table 9. Analysis of inheritance of cysteine auxotrophy and streptomycin sensitivity of the donor in MS830 x MS374 crosses.[a]

Unselected phenotype	Selected phenotype		
	329[b] Ilv^+	219 Thr^+	253 Pro^+
$CysE^-$	1.2[c]	2.7	<1
Streptomycin sensitivity	11.5	3.2	9.5

[a]60 min mating.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

from an origin near *ilv* and in a clockwise direction as O-*ilv*-*thr*-*pro*-*his*.

From the analysis of the gradient of transfer (Table 7) and kinetic studies (Fig. 5) of the MS830 x MS371 matings it was concluded that the orientation of transfer was O-*ilv*-*thr*-*his*.

The recombination frequencies of the CysE$^+$ recombinants observed from the matings of MS830 x MS90 and MS830 x MS92 were identical (Table 7). It was not possible to study the *ilv* and *thr* loci in MS92 due to its extremely slow growing nature. MS90 was useful in determining a gradient of transfer for *cysE* and *ilv* (Table 7).

Figure 6 shows the time of entry of the *cysE*$^+$ and *ilv*$^+$ genes from the MS830 x MS90 mating. It appeared that the *cysE*$^+$ gene was transferred approximately 10 min after initiation of mating regardless of whether transferred as a plasmid or chromosomal marker and the *ilv*$^+$ gene was transferred after 25 minutes.

The mating MS830 x MS390 was very useful in demonstrating a gradient of transfer of *cysE* *ilv* *thr* (Table 7). This recipient was similar to MS92, but was derived as a recombinant from an MS830 x MS374 mating and had the typical *S. pullorum* growth rate, unlike the slower growing MS92.

The analysis of the linkage data (Table 10) and kinetic studies (Fig. 7) from the MS830 x MS390 matings

Figure 5. Time of entry of various markers from MS830 x MS371 matings. MS830 was mated with MS371 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Ilv^+, Thr^+ and His^+ recombinants. The selective media were supplemented with leucine and cysteine. Uridine auxotrophy was used for counterselection.

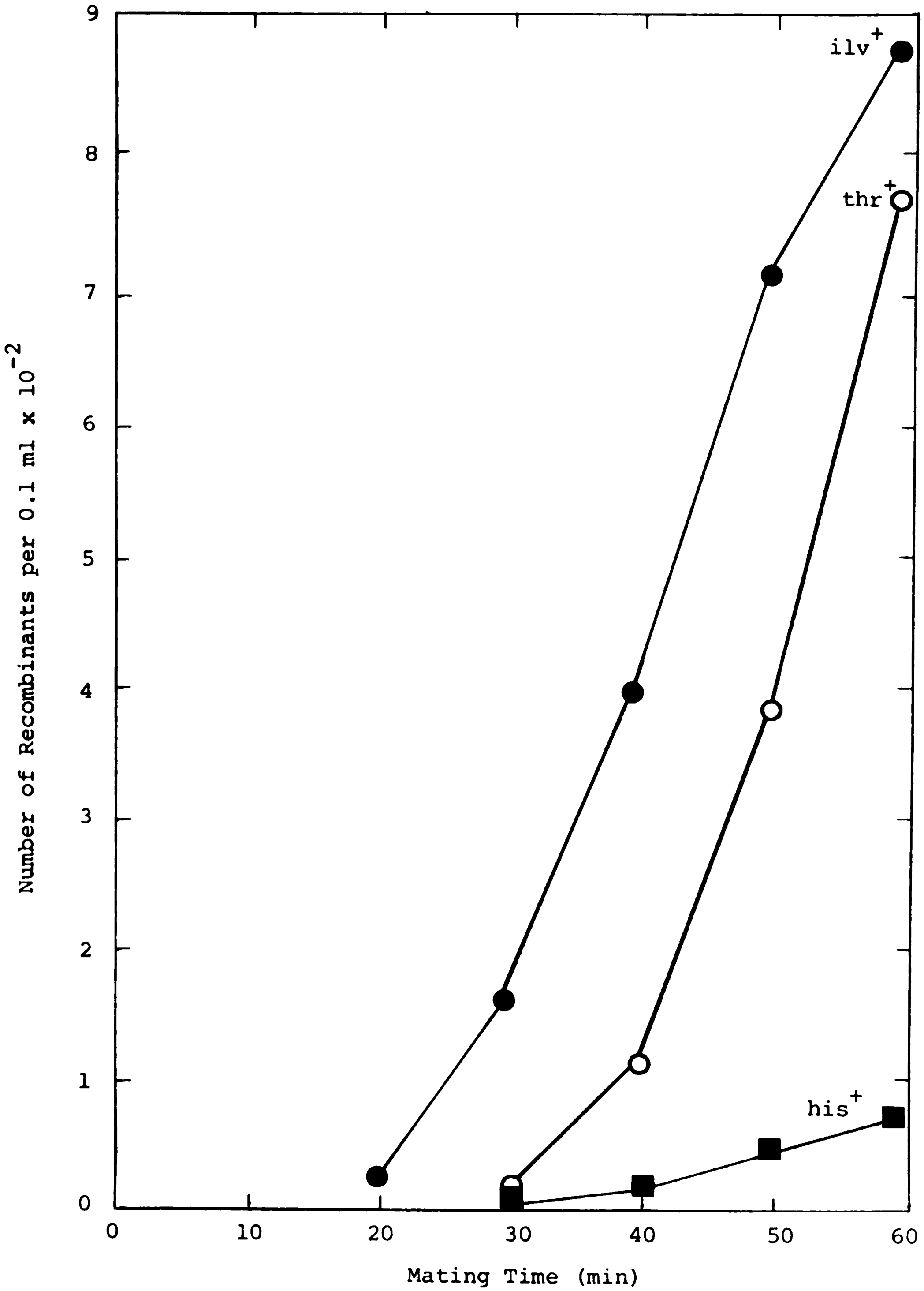
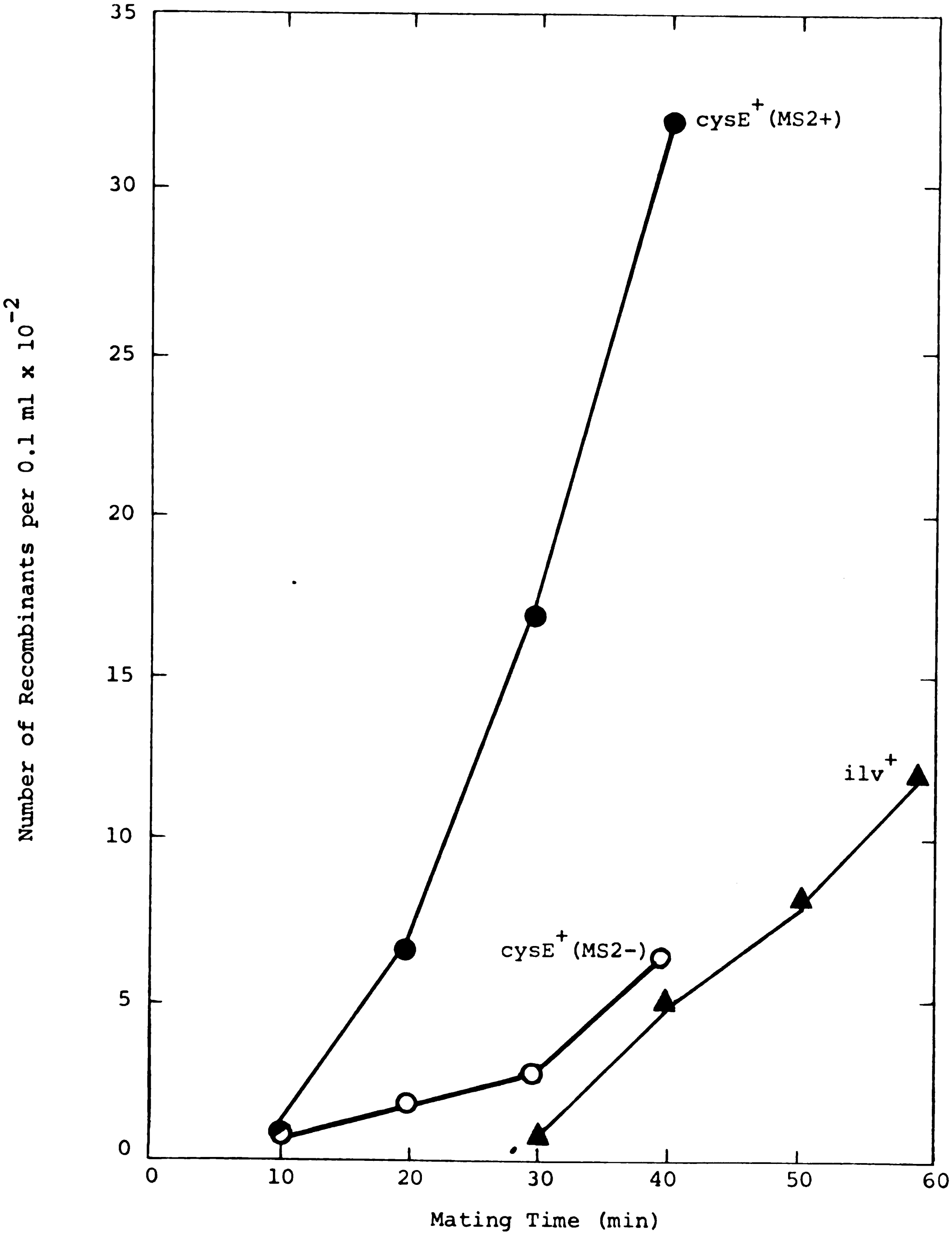

Number of Recombinants per 0.1 ml x 10-2
Mating Time (min)
ilv+
thr+
his+
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60

Figure 5

Figure 6. Time of entry of various markers from MS830 x MS90 matings. MS830 was mated with MS90 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for $CysE^+$ and Ilv^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection.



cysE+ (MS2+)
cysE+ (MS2-)
ilv+
Number of Recombinants per 0.1 ml x 10-2
Mating Time (min)
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60

Figure 6

Table 10. Analysis of inheritance of unselected donor markers in recombinants from the MS830 x MS390 crosses.[a]

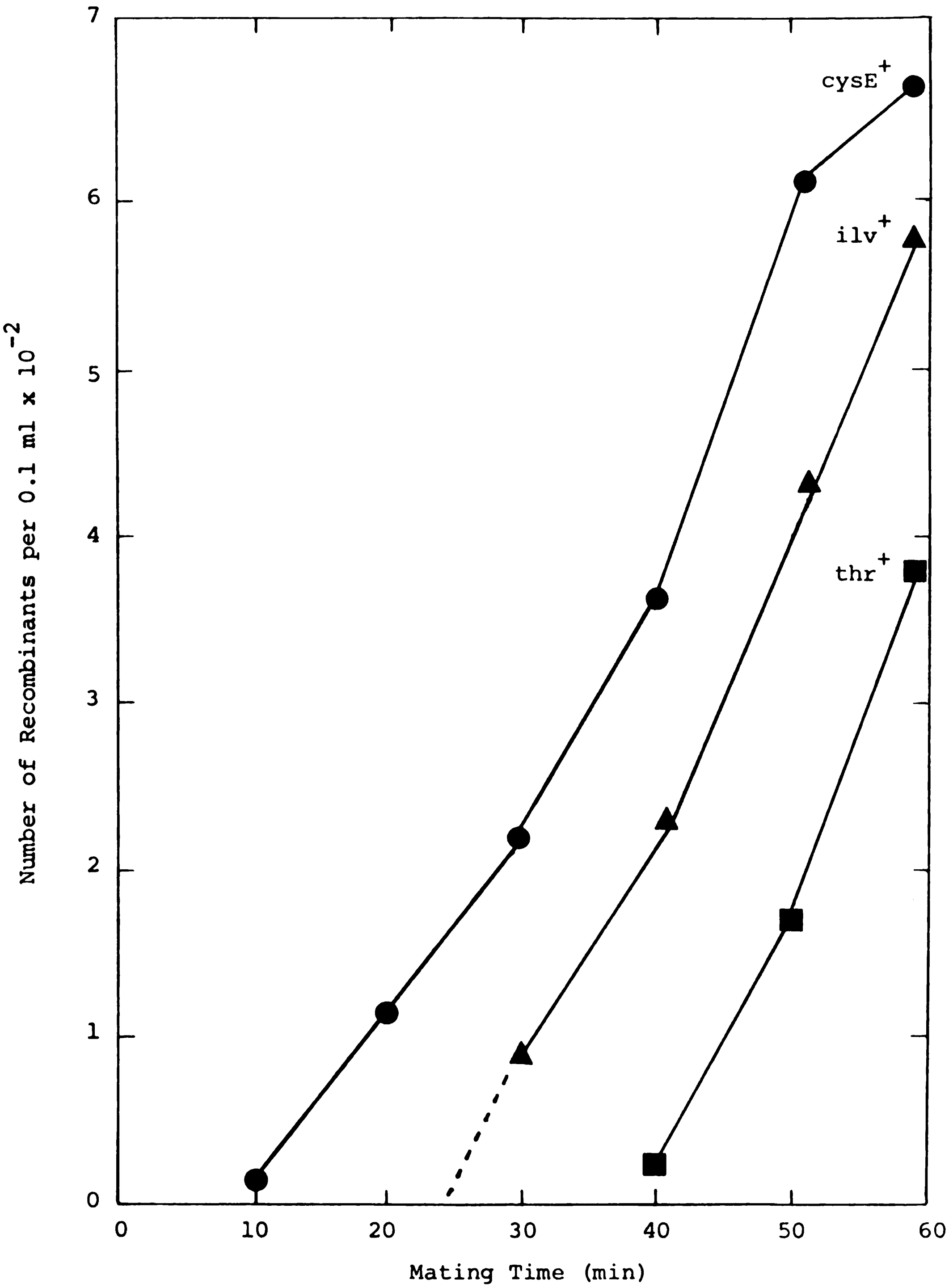
Unselected phenotype	Selected phenotype		
	200[b] $CysE^+$	196 Ilv^+	181 Thr^+
$CysE^+$	-	33.7	6.1
Ilv^+	20[c]	-	13.3
Thr^+	4.5	8.7	-

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

Figure 7. Time of entry of various markers from MS830 x MS390 matings. MS830 was mated with MS390 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for $CysE^+$, Ilv^+ and Thr^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection.



Number of Recombinants per 0.1 ml x 10^{-2}
Mating Time (min)
0
1
2
3
4
5
6
7
10
20
30
40
50
60
$cysE^+$
ilv^+
thr^+

Figure 7

led to the conclusion that the linear arrangement and orientation of transfer of the selected genes was O-cysE-ilv-thr.

The analysis of the linkage data of the MS830 x MS391 and MS392 matings (Table 11) further suggested linkage of cysE to ilv, thr and pro. Combining the data from parts A and B of Table 11, one is able to construct a linkage map as O-cysE-ilv-thr-pro.

Figure 8 is a composite of time of entry studies with MS830 and the recipients MS391 and MS392. These data confirmed the gene order based on the linkage data presented in Table 11.

Zygotic induction. MS830 was made lysogenic for P35 as described in the Materials and Methods section and designated MS8301. This donor was mated with the nonlysogenic recipient MS81 on millipore filters as described in the Material and Methods section. The apparent time of entry of the att $P35^+$ marker was at approximately 45 min after initiation of chromosomal transfer by F77 (Fig. 9).

The matings of MS830 x MS104 demonstrated a gradient of transfer of trp>cysB>his (Table 7). From the analysis of the linkage data (Table 12) and kinetic studies (Fig. 10) from the MS830 x MS104 matings, it was concluded that the linear arrangement and orientation of the selected markers was O-trp-cysB-his. Therefore, I inferred that there was also an origin of transfer for F77 between pyrD

Table 11. Analysis of inheritance of unselected donor markers in recombinants from crosses with MS830 and the *S. pullorum* recipients MS391 and MS392.[a]

A. Mating: MS830 x MS391

Unselected phenotype	Selected phenotype	
	284[b] $CysE^+$	179 Ilv^+
$CysE^+$	-	40.7
Ilv^+	23.3[c]	-
Pro^+	3.1	7.2

B. Mating: MS830 x MS392

Unselected phenotype	Selected phenotype	
	384[b] $CysE^+$	269 Thr^+
$CysE^+$	-	5.9
Thr^+	6.0[c]	-
Pro^+	1.0	1.5

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

Figure 8. Time of entry of various markers from MS830 x MS392(A) and MS830 x MS391(B) matings. The matings were done on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for $CysE^+$, Ilv^+ and Thr^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection. Symbols: MS830 x MS392-○ and □ , MS830 x MS391- ● and ■ .

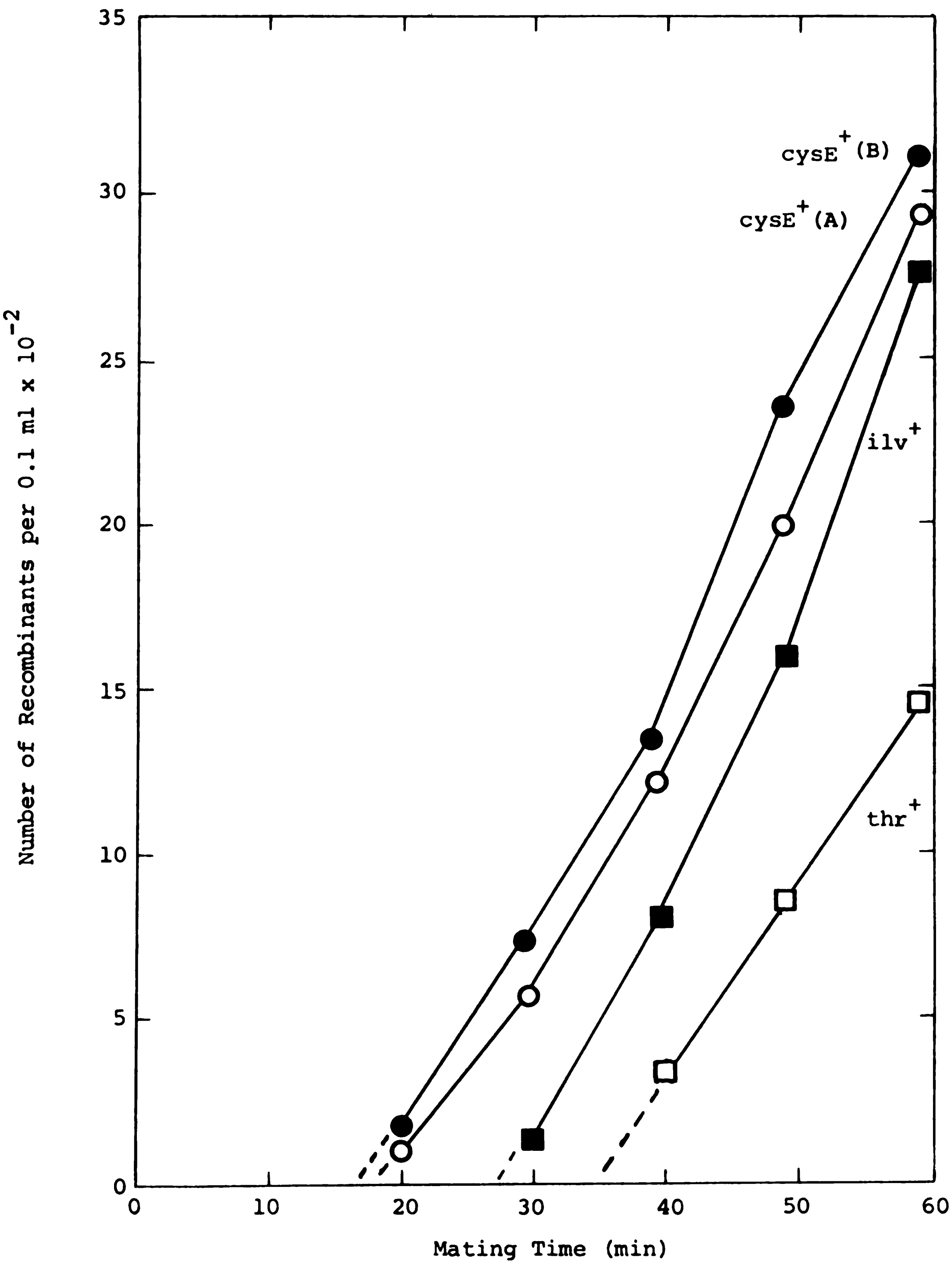

Number of Recombinants per 0.1 ml x 10^{-2}
Mating Time (min)
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
cysE$^+$(B)
cysE$^+$(A)
ilv$^+$
thr$^+$

Figure 8

Figure 9. Time of entry of _att P35_$^+$ from the MS8301 x MS81 mating. MS8301 was mated with MS81 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3×10^2 donor cells) was mixed with MS53 in an L soft agar overlay and poured over the surface of an L agar plate.

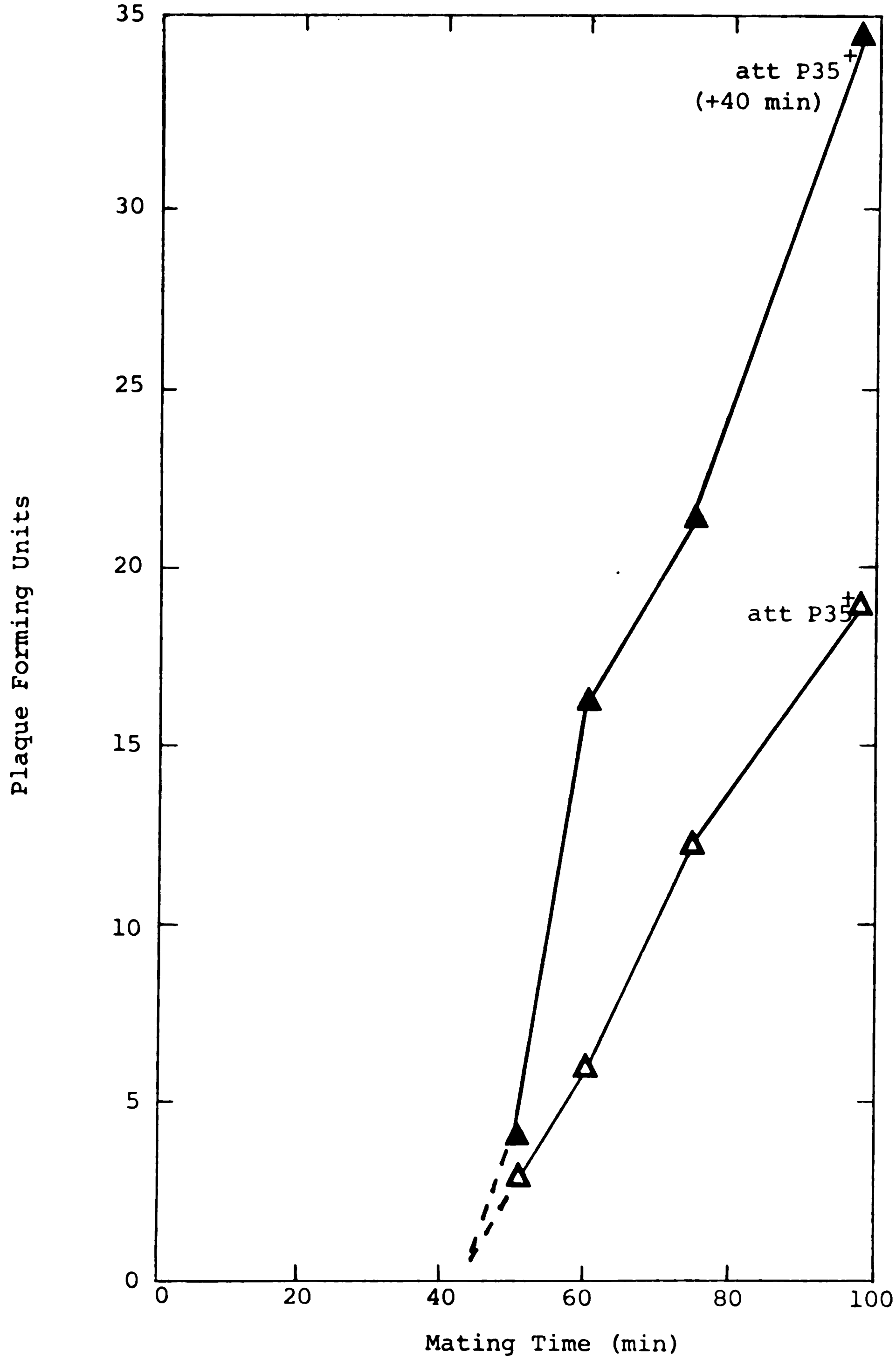

35
30
25
20
15
10
5
0
Plaque Forming Units
att P35+
(+40 min)
att P35+
0
20
40
60
80
100
Mating Time (min)

Figure 9

Table 12. Analysis of inheritance of unselected donor markers in recombinants from the MS830 x MS104 crosses.[a]

Unselected phenotype	Selected phenotype		
	153[b] Trp^+	221 $CysB^+$	229 His^+
Trp^+	-	92	3.5
$CysB^+$	98[c]	-	3.9
His^+	2	3	-

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

Figure 10. Time of entry of various markers from MS830 x MS104 matings. MS830 was mated with MS104 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Trp^+, $CysB^+$ and His^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection.

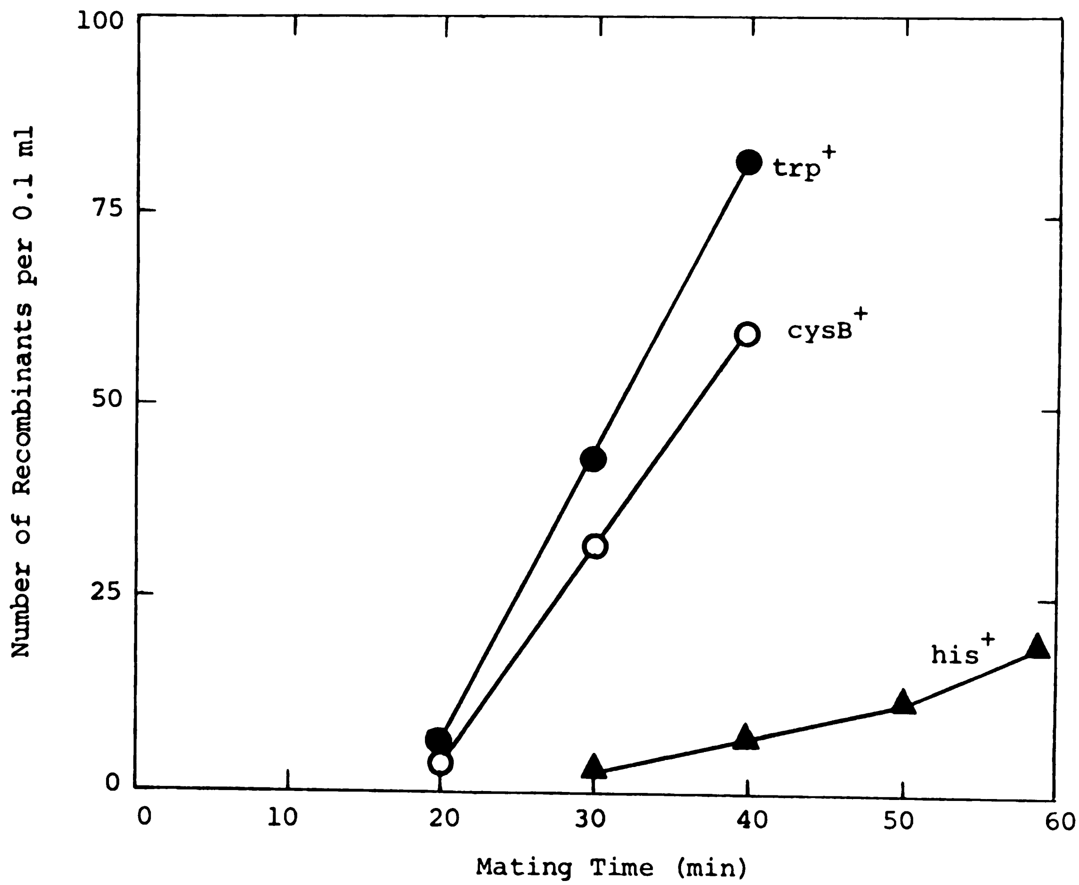

100
75
50
25
0
Number of Recombinants per 0.1 ml
trp+
cysB+
his+
0
10
20
30
40
50
60
Mating Time (min)

Figure 10

and trp cysB which transferred in the clockwise direction as O-trp-cysB-his. The high degree of linkage of cysB and trp (Table 12) was expected as it had been shown that they are co-transducible in S. pullorum (42).

Part III

Spontaneous Mutation of F77

The donor MS831 was chosen for further study after its accidental isolation. This donor strain appeared to be identical to MS8300 except that it was auxotrophic for cysE due to an apparent spontaneous mutation of the F77 cysE gene. Hereafter, this mutant transmissible plasmid will be designated F77cysE$^-$.

Matings using the donor MS831. The recombination frequencies and gradient of transfer for the various selected markers from the crosses of the donor MS831 and various S. pullorum recipients are listed in Table 7.

The gradient of transfer and recombination frequencies for the MS831 x MS369 and MS831 x MS374 matings differed considerably from those of MS830 and the same recipients (Table 7).

There were no CysE$^+$ recombinants from MS831 x MS90 and MS831 x MS92 matings.

The gene order based upon the gradient of transfer from the MS831 x MS104 matings was trp-cysB-his (Table 7).

It was evident from Table 13 that the trp and cysB genes were very closely linked but have little linkage to his and that the order of gene entry was O-trp-cysB-his (Fig. 11).

These data substantiated the data previously observed for these three genes in the MS830 x MS104 matings (Table 12, Fig. 10). Therefore, I inferred that F77cysE$^-$ transferred the host chromosome from an origin similar to or identical to the origin of transfer for F77 between pyrD and trp, also in the clockwise direction.

Part IV

Determination of a Homogeneous or Heterogeneous Donor Population and Selection for Donors with Increased Fertility

After observing the ability of the donor MS830 to transfer its chromosome from possibly two origins and at moderate frequencies, I attempted to select donor strains that might transfer at a higher frequency and/or exclusively from only one origin.

Poisson distribution test. I employed the Poisson distribution test as described in the Materials and Methods section. There was growth in 49 of 100 tubes of L broth following diluting and dispensing the MS830 culture. According to the Poisson distribution if there is no growth in 37% of the inoculated tubes, then there is on the average one bacterial cell per tube. I considered

Table 13. Analysis of inheritance of unselected donor markers in recombinants from the MS831 x MS104 crosses.[a]

Unselected phenotype	Selected phenotype		
	192[b] Trp^+	186 $CysB^+$	186 His^+
Trp^+	-	90	1.6
$CysB^+$	92[c]	-	1.6
His^+	3	3	-

[a]Uridine auxotrophy for counterselection and 60 min mating.

[b]The number of recombinants analyzed.

[c]The results are given as percent.

Figure 11. Time of entry of various markers from MS831 x MS104 matings. MS831 was mated with MS104 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Trp^+, $CysB^+$, and His^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection.

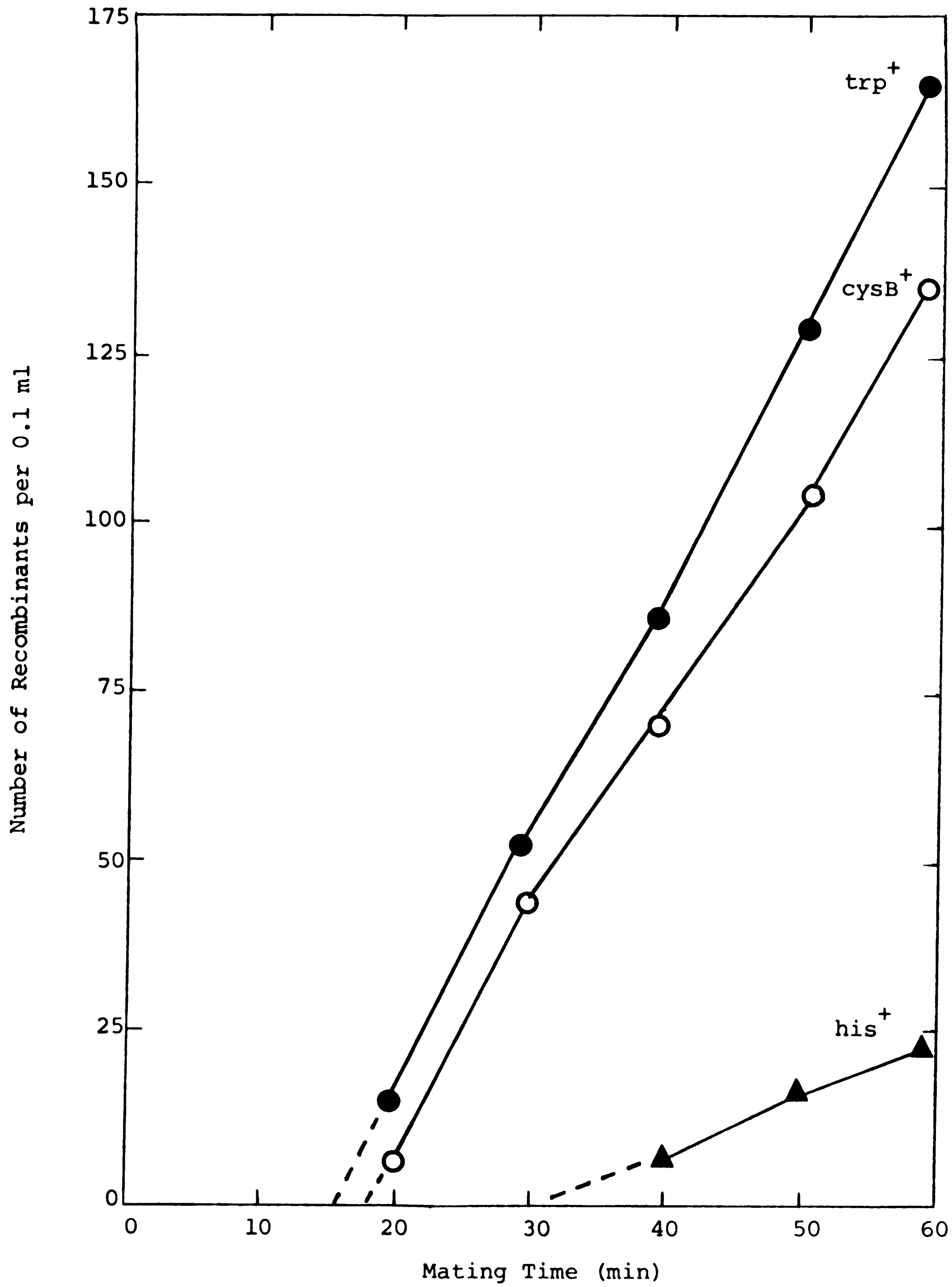

Number of Recombinants per 0.1 ml
175
150
125
100
75
50
25
0
0
10
20
30
40
50
60
Mating Time (min)
trp+
cysB+
his+

Figure 11

that the growth in the tubes inoculated with MS830 to have arisen from one bacterial cell. Each of the 49 broth cultures was tested for sensitivity to the bacteriophage MS2. Forty-eight of the 49 cultures were sensitive. These 48 cultures were cross-streak mated with the recipients MS374 and MS104 and Ilv^+ and His^+ recombinants respectively were selected.

The data collected after 48 hr of incubation at 37 C indicated that there might be three populations of donor cells: one population transferring from both origins, and two other populations with each transferring from only one of the two origins. After 96 hr of incubation at 37 C, the donor strains which appeared to transfer from only one of the two origins then appeared to be transferring from both origins. Therefore, I concluded that the donor MS830 was a homogeneous population carrying F77 which could transfer from both the origin near *cysE* and between *pyrD* and *trp*, each in the clockwise direction.

Matings using the donor MS832. The donor MS832, isolated from MS830 during the Poisson distribution experiment transferred the ilv^+ and his^+ genes initially at a frequency 4X and 3X respectively (Table 7), that previously observed with the donor MS830. Table 7 shows that during subsequent matings, the recombination frequencies for the various selected markers in mating with MS832 x MS374 dropped.

From the analysis of the linkage data (Table 14) and kinetic studies (Fig. 12) of the MS832 x MS374 matings it was concluded that the linear gene arrangement and orientation of transfer was O-*ilv*-*thr*-*pro*.

From the analysis of the gradient of transfer from the MS832 x MS104 matings (Table 7), it was concluded that the linear arrangement of these selected markers was O-*trp*-*cysB*-*his*.

The linkage data (Table 15) and kinetic studies (Fig. 13) of the MS832 x MS104 matings confirmed the suggested orientation of gene transfer as O-*trp*-*cysB*-*his*.

Electron microscopy of S. pullorum strains. The overall recombination frequencies and linkage of genetic markers from *S. pullorum* matings with chromosomal transfer mediated by F77 and F77*cysE*$^{-}$ were lower than expected (29). To see if the *S. pullorum* donors and/or recipients had any apparent cell surface structures that might be responsible for poor mating pair formation and therefore reduction in chromosomal transfer, preparations of the cultures were scanned in the electron microscope.

The recipient MS374 is shown in Fig. 14A. It looked normal with respect to gross bacterial cell surface structures.

The gross cellular appearance of the donor strain MS830 is shown in Fig. 14B. The average number of sex-pili per donor cell in *S. pullorum* is about 15. The donor MS831 and MS832 were identical in appearance to MS830.

Table 14. Analysis of inheritance of unselected donor markers in recombinants from the MS832 x MS374 crosses.[a]

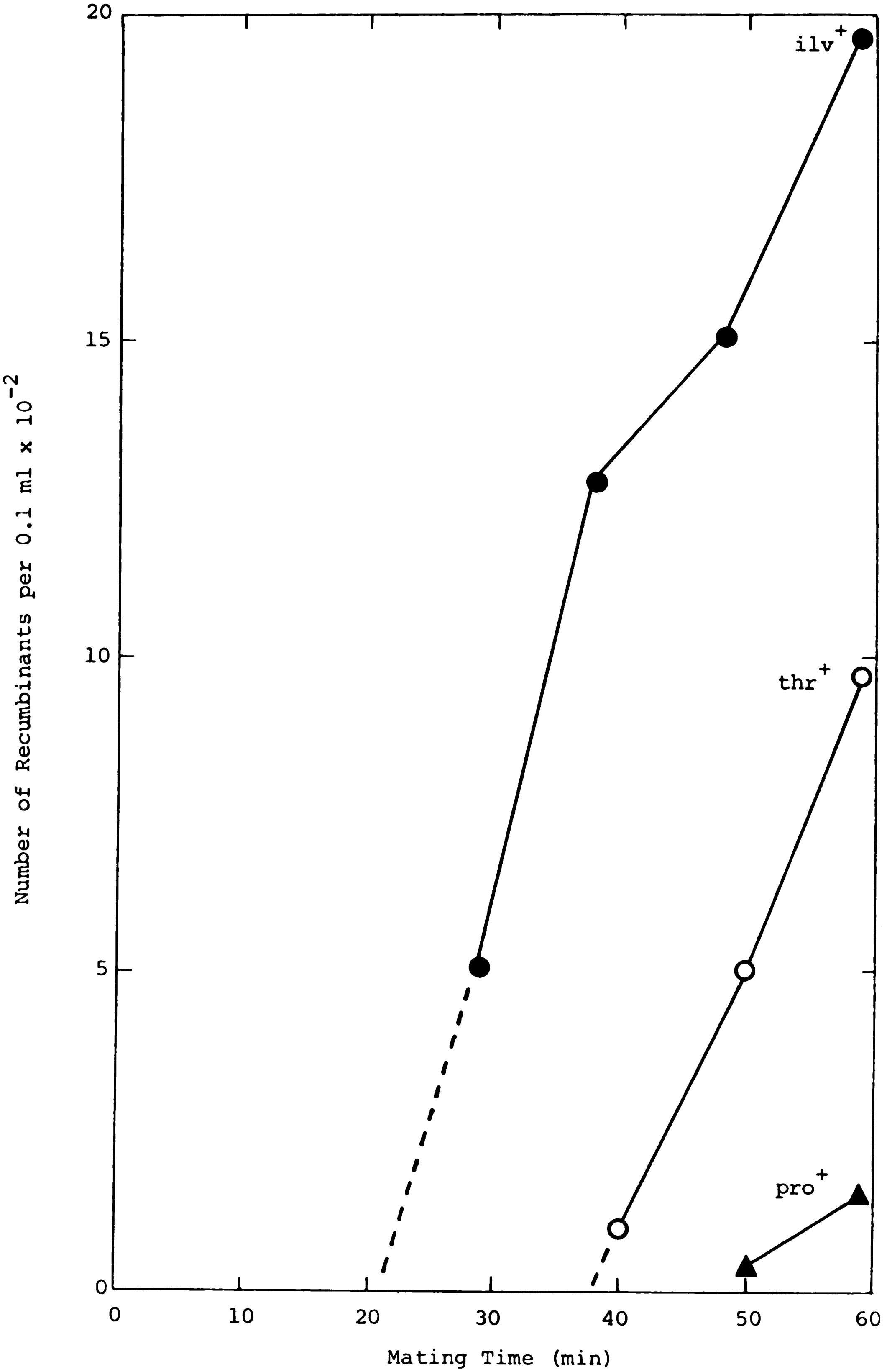
Unselected phenotype	Selected phenotype		
	212[b] Ilv^+	198 Thr^+	140 Pro^+
Ilv^+	-	17.4	6.2
Thr^+	12.6[c]	-	9.4
Pro^+	7.2	8.6	-

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results given as percent.

Figure 12. Time of entry of various markers from MS832 x MS374 matings. MS832 was mated with MS374 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Ilv^+, Thr^+ and Pro^+ recombinants. The selective media were supplemented with leucine and cysteine. Uridine auxotrophy was used for counter-selection.



20
15
10
5
0
0
10
20
30
40
50
60
Number of Recumbinants per 0.1 ml x 10^{-2}
Mating Time (min)
ilv+
thr+
pro+

Figure 12

Table 15. Analysis of inheritance of unselected donor markers in recombinants from the MS832 x MS104 cross.[a]

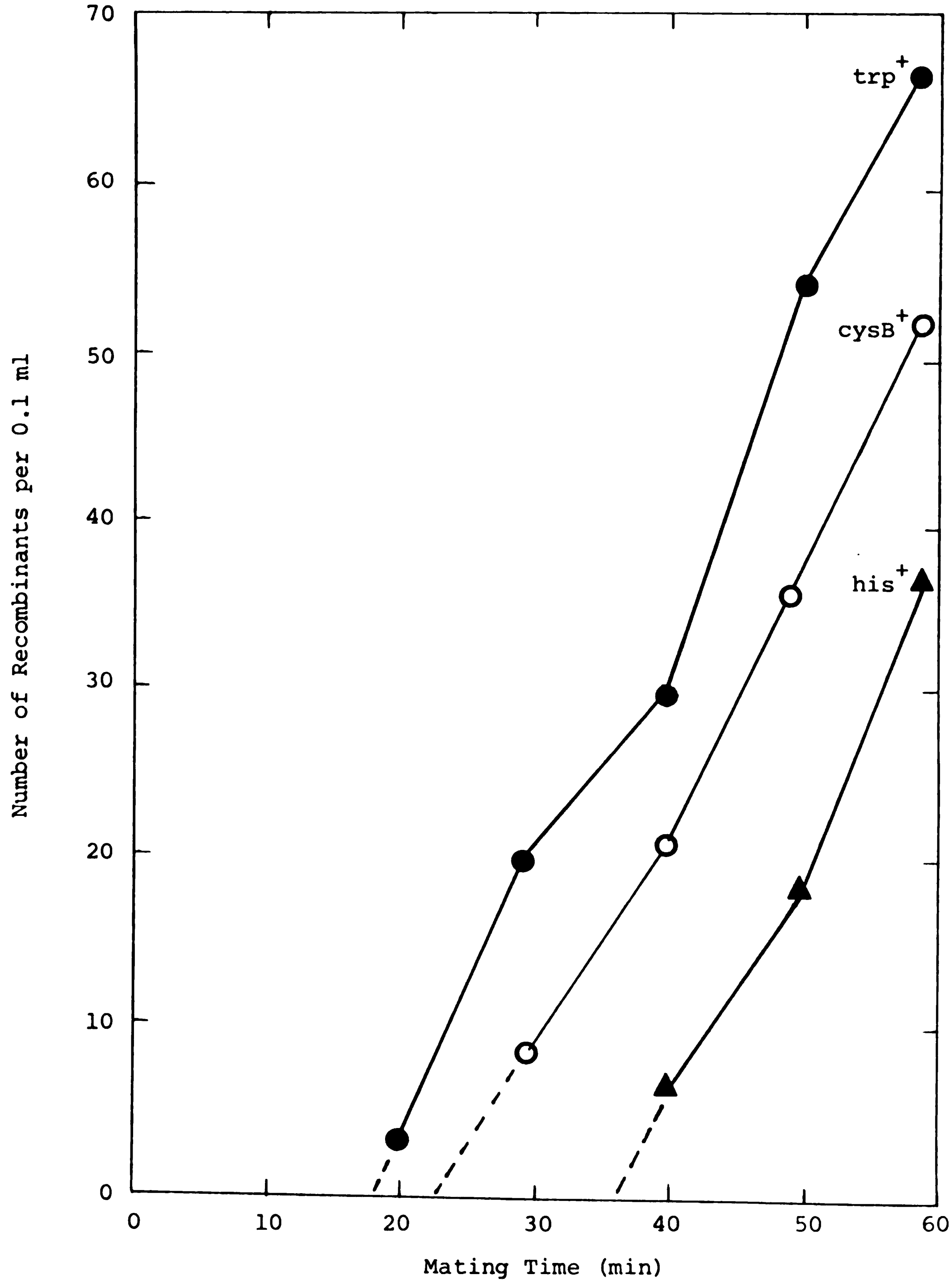
Unselected phenotype	Selected phenotype		
	114[b] Trp^+	68 $CysB^+$	53 His^+
Trp^+	-	89.7	1.9
$CysB^+$	94.7[c]	-	1.9
His^+	15.8	5.8	-

[a]Uridine auxotrophy used for counterselection and 60 min mating period.

[b]The number of recombinants analyzed.

[c]The results given as percent.

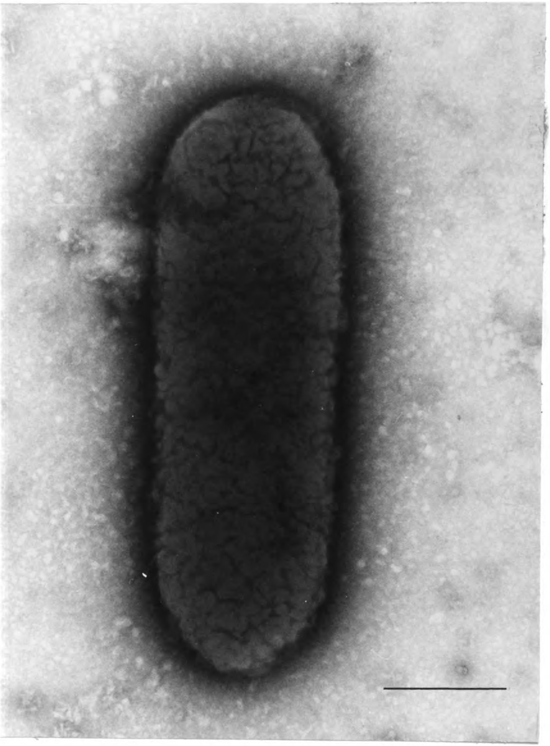
Figure 13. Time of entry of various markers from MS832 x MS104 matings. MS832 was mated with MS104 on millipore filters and transfer was interrupted at various times. A 0.1 ml of the mating suspension (3.4×10^7 donor cells) was plated at each time interval on media selective for Trp^+, $CysB^+$ and His^+ recombinants. The selective media were supplemented with leucine. Uridine auxotrophy was used for counterselection.

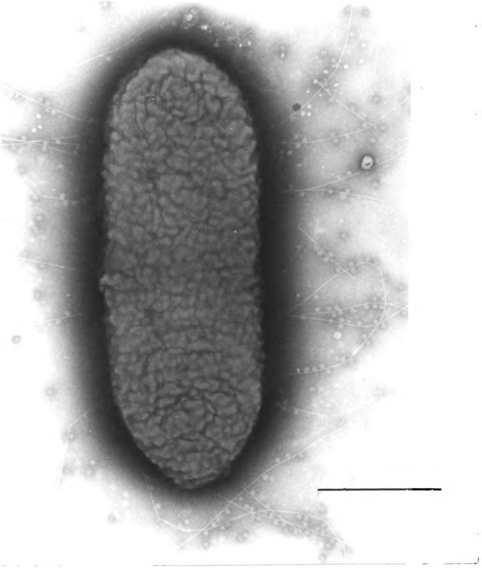


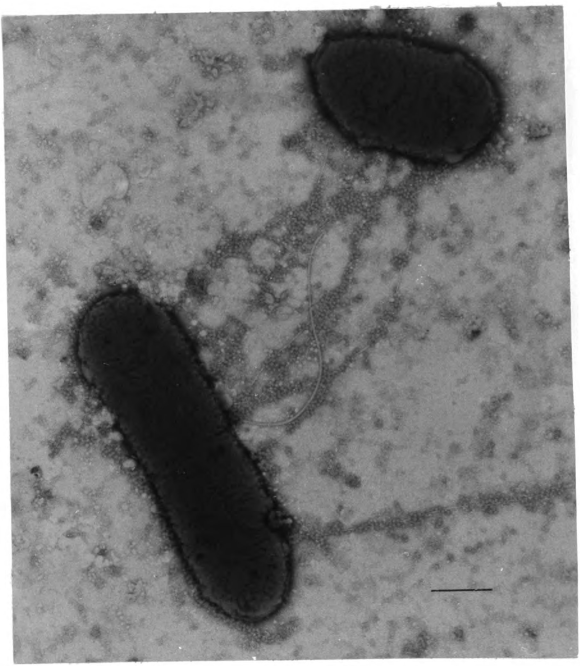
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
Number of Recombinants per 0.1 ml
Mating Time (min)
trp+
cysB+
his+

Figure 13

Figure 14. Electron micrographs of *S. pullorum* mating types. The cells were prepared for viewing by washing them off a millipore filter into sterile saline. The bar in each picture represents 0.5u. A. Recipient, MS374. Magnification is 65,000 x. B. Donor, MS830. Magnification is 65,000 x. C. Mating pair, MS830 x MS374. Magnification is 31,500 x.







The *S. pullorum* donor and recipient in Fig. 14C might actually represent effective pair formation. It appeared that many of the sex-pili of the donor make contact with the recipient cell.

In all cases, the male specific RNA bacteriophage MS2 were found adsorbing to the sex-pili of the donor cells.

Part V

Evidence of F77 Converting to a More Stable Association with the Host Chromosome

The MS8300 strain was checked regularly by the cross-streak method for its ability to transfer markers and from each test the isolate transferring at the highest frequency was chosen as the donor for mating experiments. The recombinants from these matings were analyzed for their sensitivity to MS2. Those recombinants receiving F77 were sensitive and indicated that F77 was in the autonomous state. Upon stable association or integration of F77 and the host chromosome, the recombinants would only receive the sex factor as the terminal marker characteristic of Hfr mediated chromosomal transfer. Integration of F77 should lead to a reduction in the number of MS2 sensitive recombinants when selection was for a marker transferred very early. Concomitant with this transition, one should observe an increase in frequency of selected donor markers in recombinants and a stability of donor ability in the donor strain studied.

It was pointed out in the Materials and Methods section that the S. pullorum donor strains harboring F77 were quite stable with regard to donor ability measured by MS2 sensitivity.

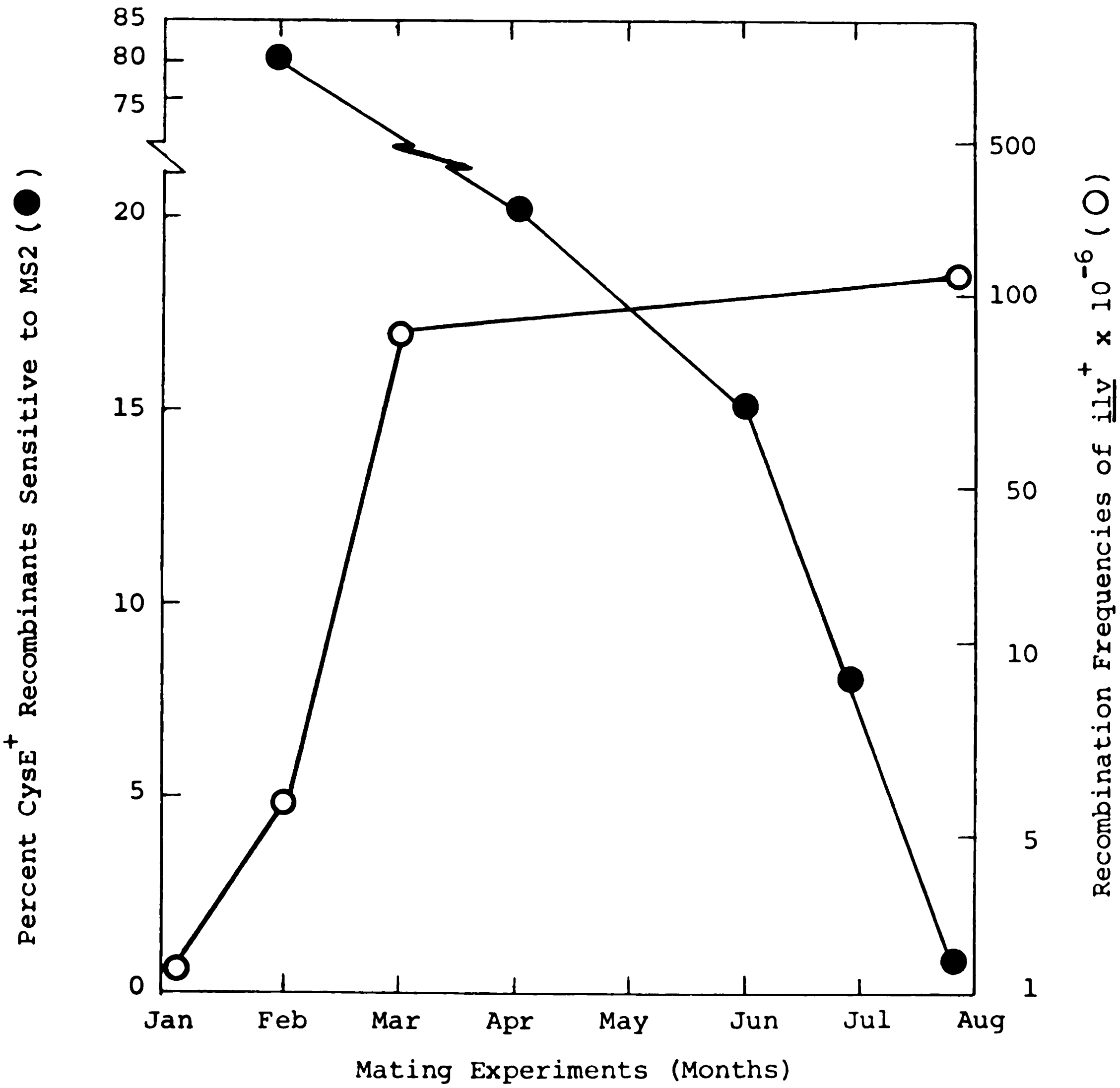
Figure 15 shows the decreasing percentage of selected $CysE^+$ recombinants remaining MS2 sensitive and an increasing relative recombination frequency of Ilv^+ recombinants during an eight-month period study of MS8300 x MS90 matings.

Part VI

Isolation and Characterization of Plasmid DNA

Lysates prepared from S. pullorum donor strains MS830 and MS831, recipient strain MS83 and S. typhimurium SA532 and SA1466, a donor and recipient, respectively, were subjected to equilibrium centrifugation in a solution of CsCl-EtBr to determine whether the F77 DNA could be separated from chromosomal DNA. With lysates of both the donor and recipient strains of S. pullorum a more dense sedimenting fraction characteristic of closed circular DNA (80) and a less dense fraction characteristic of open circular DNA and linear DNA were observed (Fig. 16A). With the lysates of the donor strain of S. typhimurium, SA532, similar fractions were observed (Fig. 16B) but with

Figure 15. Evidence for F77 converting to a more stable association with the host chromosome. The selected recombinants from the MS8300 x MS90 matings conducted over an eight-month period were analyzed at various times for the transfer of F77 and the donor marker _ilv_$^{+}$ gene.



85
80
75
20
15
10
5
0
500
100
50
10
5
1
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Mating Experiments (Months)
Percent CysE+ Recombinants Sensitive to MS2 (●)
Recombination Frequencies of ilv+ x 10-6 (○)

Figure 15

Figure 16. Isolation of plasmid DNA from *S. pullorum* MS35 derivatives and *S. typhimurium*. Three ml of cleared lysate of the various strains labeled with ^{3}H-thymidine or ^{14}C-thymidine were centrifuged in CsCl-EtBr dye buoyant density gradients in a Type 50 rotor at 44,000 rpm for 30 hr at 15 C. Fractions were collected and 5 ul samples spotted on filter paper squares, washed in TCA, ethanol and ether and counted for radioactivity. Fractions 20-27 containing plasmid DNA were pooled and dialyzed in the dark against TES buffer at 5 C. (A) *S. pullorum* MS83, MS8300, MS830 and MS831, —■—, (B) *S. typhimurium* SA532, —■—□—, (C) *S. typhimurium* SA1466, —□— . Symbols: ^{3}H-thymidine —■—, ^{14}C-thymidine —□— .

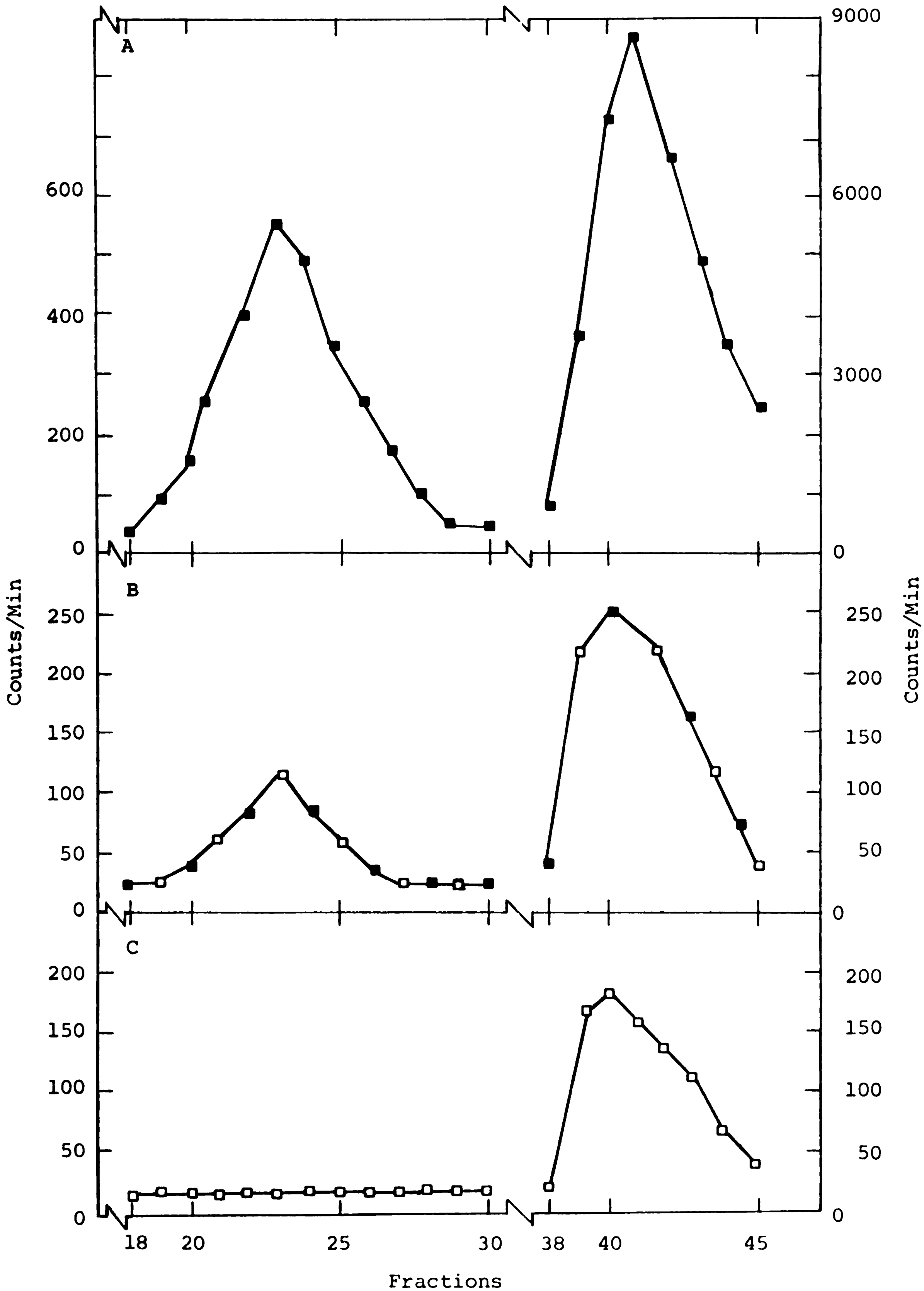

A
B
C
600
400
200
0
250
200
150
100
50
0
200
150
100
50
0
9000
6000
3000
0
250
200
150
100
50
0
200
150
100
50
0
Counts/Min
Counts/Min
18
20
25
30
38
40
45
Fractions

Figure 16

the recipient strain of S. typhimurium, SA1466, the more dense fraction was not present (Fig. 16C).

The fractions corresponding to higher density of each strain were separately pooled, dialyzed and layered on 20-31% neutral sucrose gradients and subjected to centrifugation to ascertain whether there were molecules of different sedimentation coefficients in these fractions. As expected, the donor strain of S. typhimurium carried a closed circular DNA molecule (Fig. 17). Two peaks of radioactivity appeared in the denser material from the S. pullorum recipient MS83 (Fig. 18A), and the donor MS831 (Fig. 18C). The results with MS83 were expected since two plasmids, PO-1 and PO-2, which have s values of 17 and 65 respectively, are found in another strain of S. pullorum, MS53 (73).

The fact that a PO-2-like plasmid was present in MS831 (Fig. 18C) but absent in MS830 (Fig. 18B) surprised me. Since infection of MS53 with phage P35 resulted in the loss of the PO-2 plasmid (W. L. Olsen and D. E. Schoenhard, Bacteriol. Proc., p. 46, 1971), MS83 and MS831 were infected with phage P35 to find out whether the PO-2-like plasmid would be lost. A peak of radioactivity corresponding to the PO-2-like plasmid was observed in a sedimentation profile made from pycnographically separated DNA of MS831; similar results were obtained with the recipient MS83 (data not shown). Therefore, neither the F77$cysE^-$ factor nor phage P35

Figure 17. Neutral sucrose gradient of plasmid DNA from *S. typhimurium* SA532. A 0.3 ml sample of ^{14}C labeled pooled plasmid DNA from the CsCl-EtBr gradient was layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW 50L rotor at 50,000 rpm for 90 min at 15 C. Fractions were collected directly onto filter paper squares and washed in TCA, ethanol and ether and counted for radioactivity.

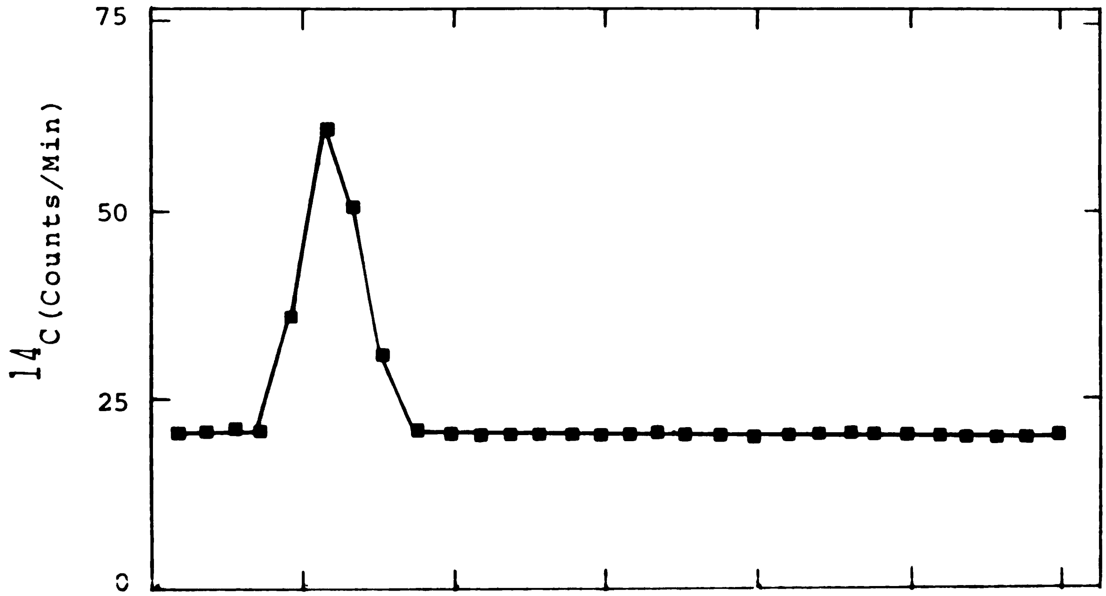


Figure 17

Figure 18. Neutral sucrose gradient of plasmid DNA from *S. pullorum* MS35 derivatives. A 0.15 ml sample of ^3H labeled pooled plasmid DNA from the CsCl-EtBr gradient was layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW 50L rotor at 50,000 rpm for 90 min at 15 C. Fractions were collected directly onto filter paper squares and washed in TCA, ethanol and ether and counted for radioactivity. (A) MS83, (B) MS830, (C) MS831.

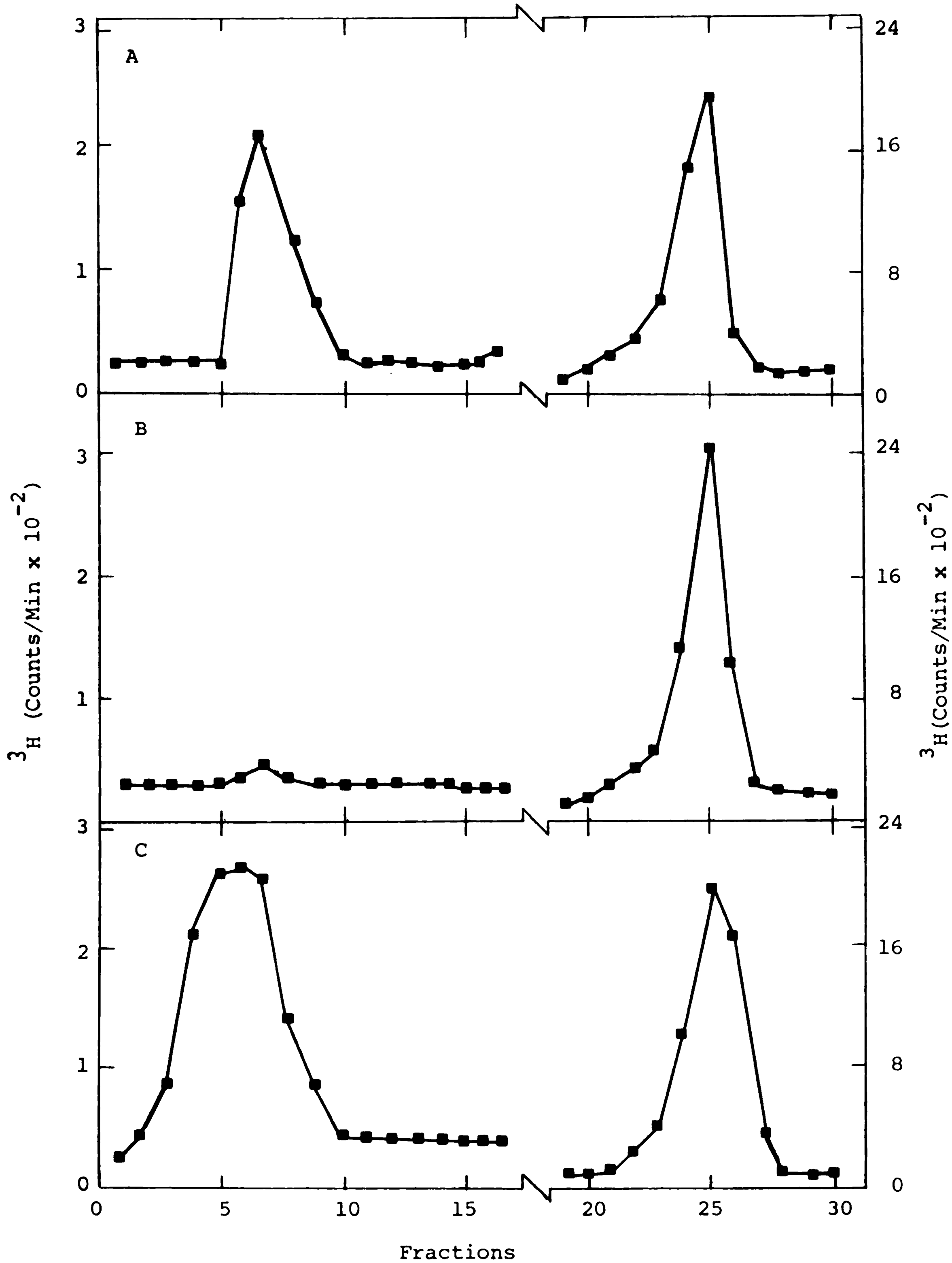

A
B
C
3
2
1
0
24
16
8
0
^3H (Counts/Min x 10^{-2})
^3H(Counts/Min x 10^{-2})
0
5
10
15
20
25
30
Fractions

Figure 18

excluded the PO-2 plasmid, but when F77 became associated with the host chromosome in MS830, the PO-2-like plasmid was lost.

In the sedimentation profiles of MS83 and MS831 it was observed that the area underneath the peak of the rapidly sedimenting plasmid was larger with the material from MS831 (Fig. 18C) than MS83 (Fig. 18A). Resolution of the broader peak of MS831 was tried by fractionating the material into smaller samples. A definite shoulder in the faster sedimenting material was observed (Fig. 19A). A new MS8300 donor strain was isolated as before. The sedimentation profiles of pycnographically separated DNA from MS8300 (Fig. 19B and 20A) were very similar to the ones found with MS831 (Fig. 18C and 19A).

A series of reconstruction experiments were done to show that the PO-2-like plasmid of MS831 was a composite of F77*cysE*$^-$ and the PO-2 plasmid. Cosedimentation of F77 DNA isolated from *S. typhimurium* SA532 with plasmid DNA from MS83 resulted in the F77 DNA sedimenting with a greater velocity than the PO-2 plasmid DNA (Fig. 21A). An s value of 70 was calculated for F77 (17) corresponding to a molecular weight of 51 x 10^6 daltons (9). Cosedimentation of F77 DNA isolated from *S. typhimurium* SA532 with plasmid DNA isolated from MS830 (Fig. 21B) confirmed the absence of PO-2 plasmid or F77 DNA. Finally cosedimentation of F77 DNA isolated from *S. typhimurium* SA532

Figure 19. Neutral sucrose gradient of plasmid DNA from *S. pullorum* MS35 derivatives. A 0.15 ml sample of ^{3}H labeled pooled plasmid DNA from the CsCl-EtBr gradient was layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW 50L rotor at 50,000 rpm for 90 min at 15 C. Fractions were collected directly onto filter paper squares and washed in TCA, ethanol and ether and counted for radioactivity. (A) MS831, (B) MS8300.

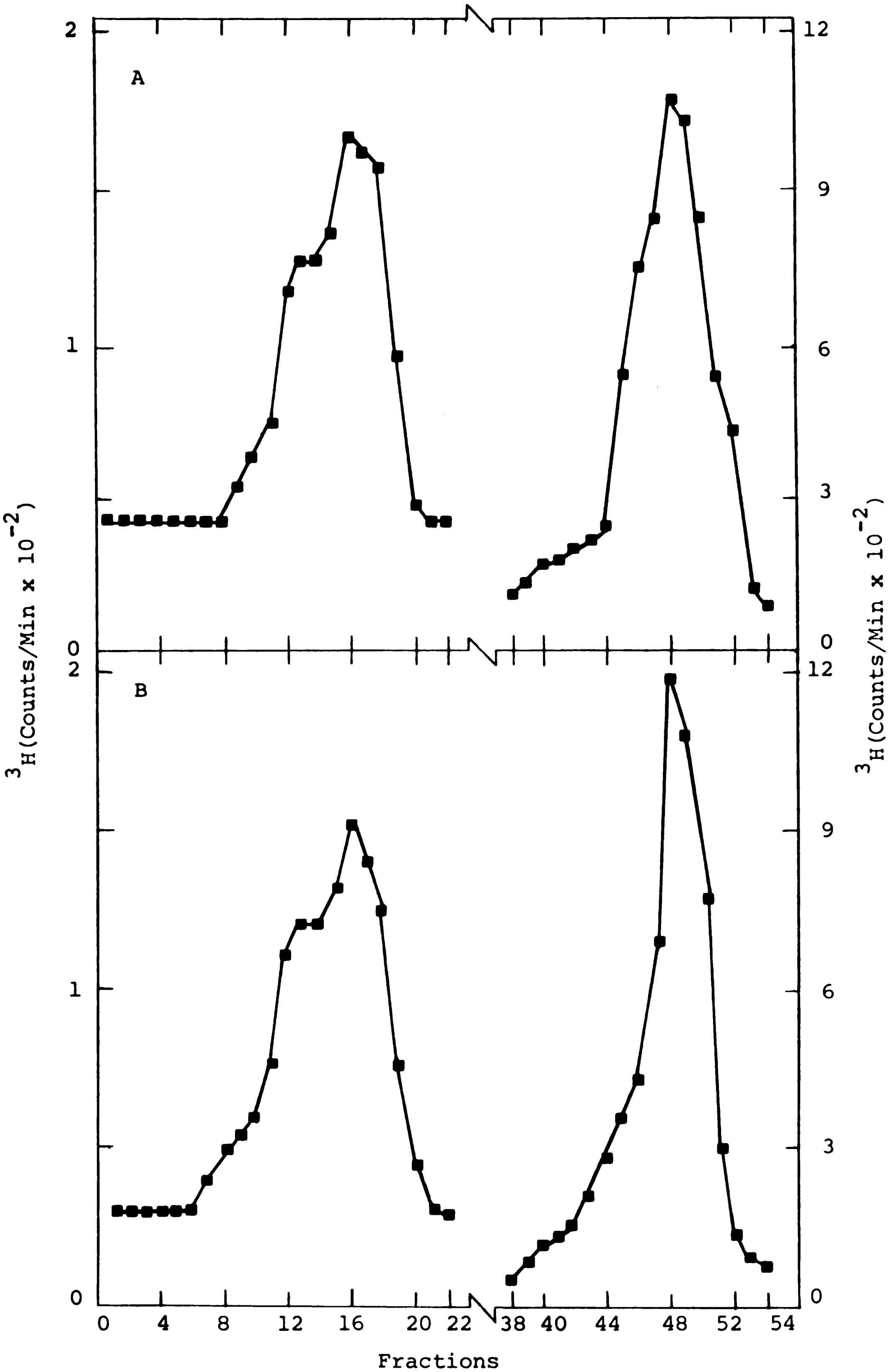

A
B
2
1
0
12
9
6
3
0
^{3}H(Counts/Min x 10^{-2})
^{3}H(Counts/Min x 10^{-2})
0 4 8 12 16 20 22 38 40 44 48 52 54
Fractions

Figure 19

Figure 20. Neutral sucrose gradient of plasmid DNA from *S. pullorum* and *S. typhimurium*. The samples of labeled pooled plasmid DNA from the CsCl-EtBr gradient were layered on a 20-31% neutral sucrose gradient. The gradient was centrifuged in an SW 50L rotor at 50,000 rpm for 90 min at 15 C. Fractions were collected directly onto filter paper squares and washed in TCA, ethanol and ether and counted for radioactivity. (A) *S. pullorum* MS8300, —■— (B) *S. pullorum* MS8300, —■—, + *S. typhimurium* SA532, —□—, (C) *S. pullorum* MS831, —■—, + *S. typhimurium* SA532, —□—. Symbols: ^{3}H-thymidine —■—, ^{14}C-thymidine —□—.

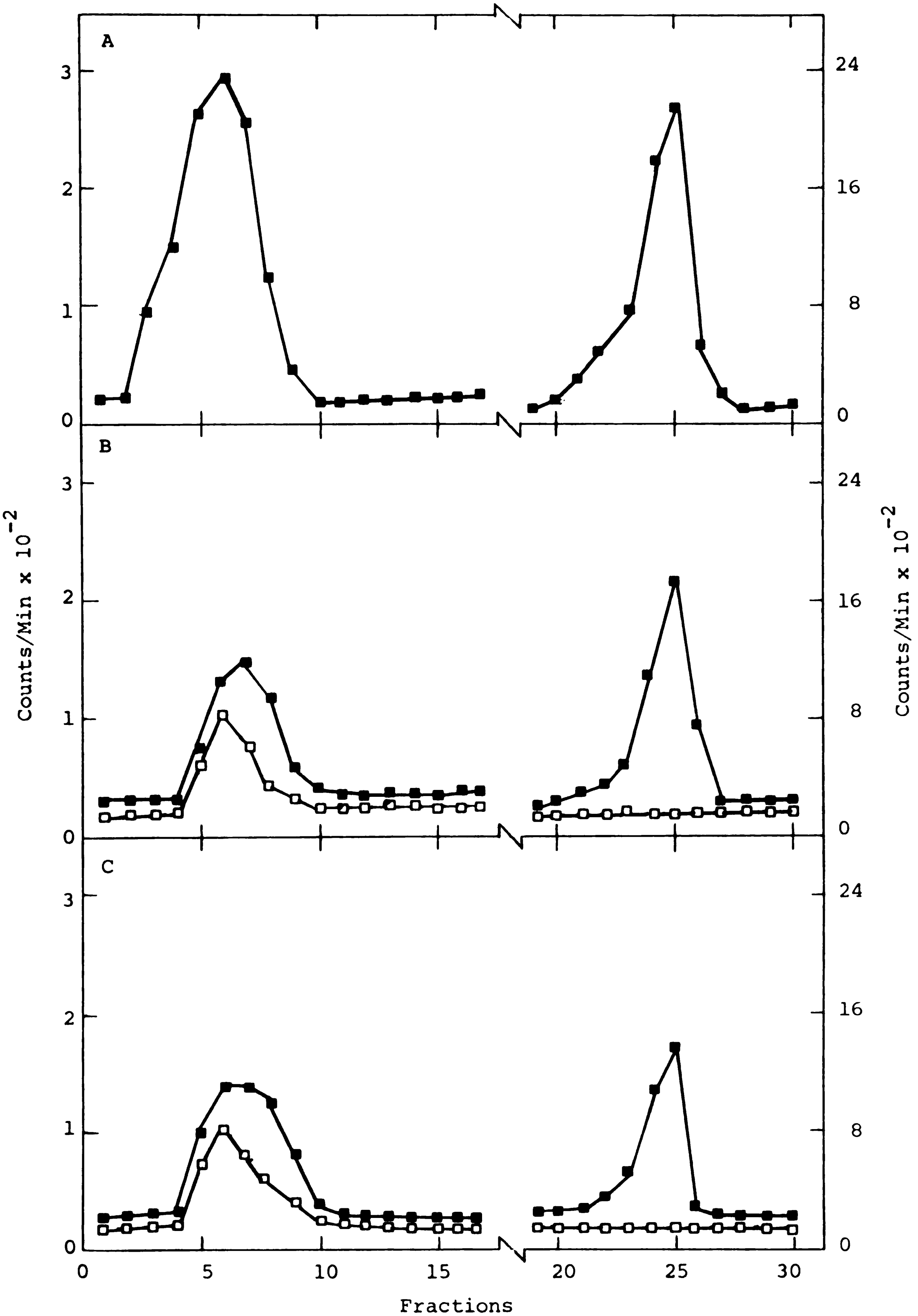

A
B
C
Counts/Min x 10^{-2}
Counts/Min x 10^{-2}
0
1
2
3
0
8
16
24
0
5
10
15
20
25
30
Fractions

Figure 20

Figure 21. Neutral sucrose gradient of plasmid DNA from *S. pullorum* and *S. typhimurium*. The samples of labeled pooled plasmid DNA from the CsCl-EtBr gradient was layered on a 20-31% nuetral sucrose gradient. The gradient was centrifuged in an SW 50L rotor at 50,000 rpm for 90 min at 15 C. Fractions were collected directly onto filter paper squares and washed in TCA, ethanol and ether and counted for radioactivity. (A) *S. pullorum* MS83, —■— , + *S. typhimurium* SA532, —○— , (B) *S. pullorum* MS830, —■— , + *S. typhimurium* SA532, —○— . Symbols: ^{3}H-thymidine —■—, ^{14}C-thymidine —○— .

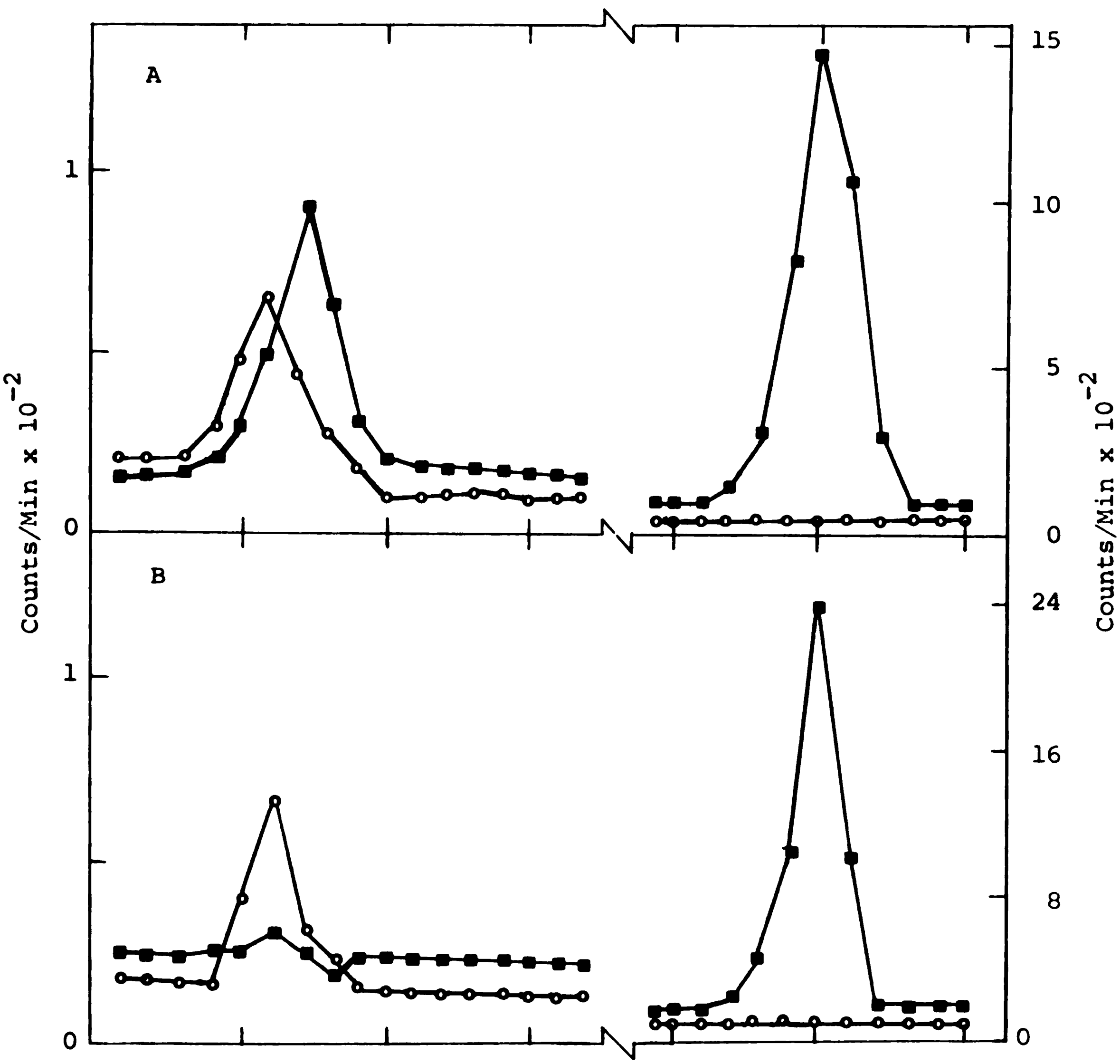


Figure 21

with PO-2-like plasmid DNA from MS831 and MS8300 resulted in the F77 DNA sedimenting with the leading edge of the broad PO-2-like plasmid DNA peak with either MS8300 (Fig. 20B) or MS831 (Fig. 20C).

A comparison of total counts of radioactivity in plasmid DNA determined from placing an aliquot portion of the cleared lysate of MS83 and MS8300 or MS831 separately onto a neutral sucrose gradients to the total radioactivity incorporated into cellular DNA, indicated that there existed approximately 20% and 24%, respectively, of the total DNA as extrachromosomal plasmid DNA in *S. pullorum*.

There was about 8X as much plasmid PO-1 DNA as PO-2 DNA. Therefore, there were about 170 copies of plasmid PO-1 for each copy of the PO-2 plasmid per cell. It appeared that there were 1-2 copies of the F77 and F77*cysE*$^-$ factor per MS8300 and MS831 respectively.

DISCUSSION

Part I

Counterselection of Donors Carrying F77

The recipient MS83 was chosen as the host for carrying F77 because it has a very stable *pyrD1* mutation which maps in the region between *pro* and *trp* in *S. pullorum*, as in *S. typhimurium*. However, the map position of *pyrD1* limits its use to short mating periods to avoid coinheritance of this marker. The control mating, MS901 x MS83, indicates that *pyrD1* is not located within the first 60 minutes of an origin of transfer for F77.

Part II

Origins of Transfer and Genetic Loci Mapped with Donors Carrying F77

Matings using the donor MS830. From the analysis of the gradient of transfer, linkage and kinetic data of the MS830 x MS374 matings (Table 7, Table 8, and Fig. 4), I infer that F77 transfers the *S. pullorum* host chromosome from the same origin and with the same orientation: O-*ilv*-*thr*-*pro*, as it transfers the *S. typhimurium* chromosome (Fig. 2). This suggests that the origin of F77 transfer in S. pullorum is not as seen in Fig. 1 (42) but in the same relative position as the origin in S. typhimurium (Fig. 2).

Recall that the genotype of MS830 is leu-1 cysE1 pyrD1/F-cysE^{+} rfa^{-} pyrE^{+}, and when streptomycin sensitive or CysE1 recombinants are selected, a small number are streptomycin sensitive and few are cysteine auxotrophs. Thus, the use of streptomycin to counterselect the donor is futile.

The gradient of transfer of the MS830 x MS369 matings (Table 7), O-ilv-thr-pro-his, is like that described for the MS830 x MS374 matings (Table 7, Table 8, and Fig. 4.). Since pyrD1 is used for counterselection, the His^{+} recombinants must result from either counterclockwise transfer from the origin of F77 transfer in S. pullorum, or from a second origin of transfer.

The kinetic data from the MS830 x MS371 matings (Fig. 5) indicate that F77 may be able to transfer the chromosome from one origin in two directions or from two different origins in S. pullorum. Therefore, the Ilv^{+} and Thr^{+} recombinants can result from clockwise chromosomal transfer as in S. typhimurium, and the His^{+} recombinants from either counterclockwise chromosomal transfer from that same origin or from another origin.

The results from the MS830 x MS90 (Fig. 6) matings indicate that cysE^{+} is the most proximal marker transferred by F77. It has the same time of entry, 10 min, whether it is transferred as the plasmid or as a chromosomal marker. This is about the same as reported by Godfrey (42).

I conclude from the gradient of transfer, linkage and kinetic data analysis of the MS830 x MS390 matings (Table 7, Table 10, and Fig. 7) that the cysE, ilv and thr genes of S. pullorum are in the same relative position as they are in S. typhimurium. It appears that F77 transfers the S. pullorum chromosome from the same origin and with the same orientation as in S. typhimurium.

From the analysis of the linkage and kinetic data from the MS830 x MS391 and MS830 x MS392 matings (Table 11 and Fig. 8) it is possible to construct a linkage map for S. pullorum as O-cysE-ilv-thr-pro, with these genes in the same relative position as in S. typhimurium (Fig. 2).

Zygotic induction. Figure 9 shows that the relative time of entry of the att $P35^+$ locus is 45 min after initiation of chromosomal transfer by F77. Since P35 antiserum was not used to adsorb free P35 released from the P35 lysogenic donor MS8301, the data in Fig. 9 can be interpreted in either one of two ways: (1) the increase in P35 titer reflects the actual transfer of the prophage P35 which has its attachment site near the pro locus which is transferred after 45 min of mating and is analogous to att $P22^+$ which maps between proC and proA in S. typhimurium, or (2) the increase in the P35 titer is simply the result of a one step growth curve resulting from free P35 phage infection of MS81, which happens to have a latent period of about 45 min.

The analysis of the gradient of transfer, linkage and kinetic data of the MS830 x MS104 matings (Table 7, Table 12, and Fig. 10) leads to the conclusion that F77 can also transfer from an origin between pyrD and trp in the clockwise direction resulting in a gradient of transfer of O-trp-cysB-his, which confirms the conclusion of Godfrey (42) who inferred that trp cysB are inverted in S. pullorum compared to S. typhimurium. Therefore, the previously observed S. pullorum His^{+} recombinants may have resulted only from transfer from this origin between pyrD and trp instead of from a random origin or by counter-clockwise transfer from an origin of two directional transfer near cysE.

Therefore, it appears that F77 has the ability to transfer the S. pullorum chromosome from at least two different origins on opposite sides of the host chromosome and both in the clockwise direction. The primary origin corresponds to the origin of transfer mediated by F77 at 116 min on the S. typhimurium chromosome map with the secondary origin corresponding to a region near 45 min on the map. This suggests a situation analogous to that reported by Clark (22) for an E. coli donor with two integrated F factors transferring from two independent origins, both in the clockwise direction, but only from one origin at a time. At this time neither an appropriate donor with counterselection by a very distal marker nor a suitable multiple auxotrophic recipient is available to determine if in fact MS830 has two integrated sex factors. Also, it may be that F77 integrates or associates initially at one of the two origins, but gives rise either to a subpopulation which transfers from the second origin or capable of transferring from both origins. An Hfr population of E. coli which gives rise to a subpopulation of Hfr donors transferring from an independent, separate origin, with the opposite orientation of transfer has been described (61). On the S. typhimurium chromosome there are two origins of transfer in opposite directions near the 47 min region (83). One origin is for an Hfr transferring in the counterclockwise direction, the other origin is for an F-prime and an Hfr transferring in the clockwise direction. Since the chromosomes of S. typhimurium and S. pullorum appear to be similar, it is possible that S. pullorum strains may have similar chromosomal irregularities or nucleotide sequence which have sufficient homology with the cysE$^+$ rfa$^-$ pyrE$^+$ portion of F77 or a sfa locus with sufficient homology with the F portion of F77 to allow reciprocal crossovers and integration for transfer from the second origin between the pyrD and trp loci.

Figure 22 shows a comparison of the revised linkage map of S. pullorum with S. typhimurium.

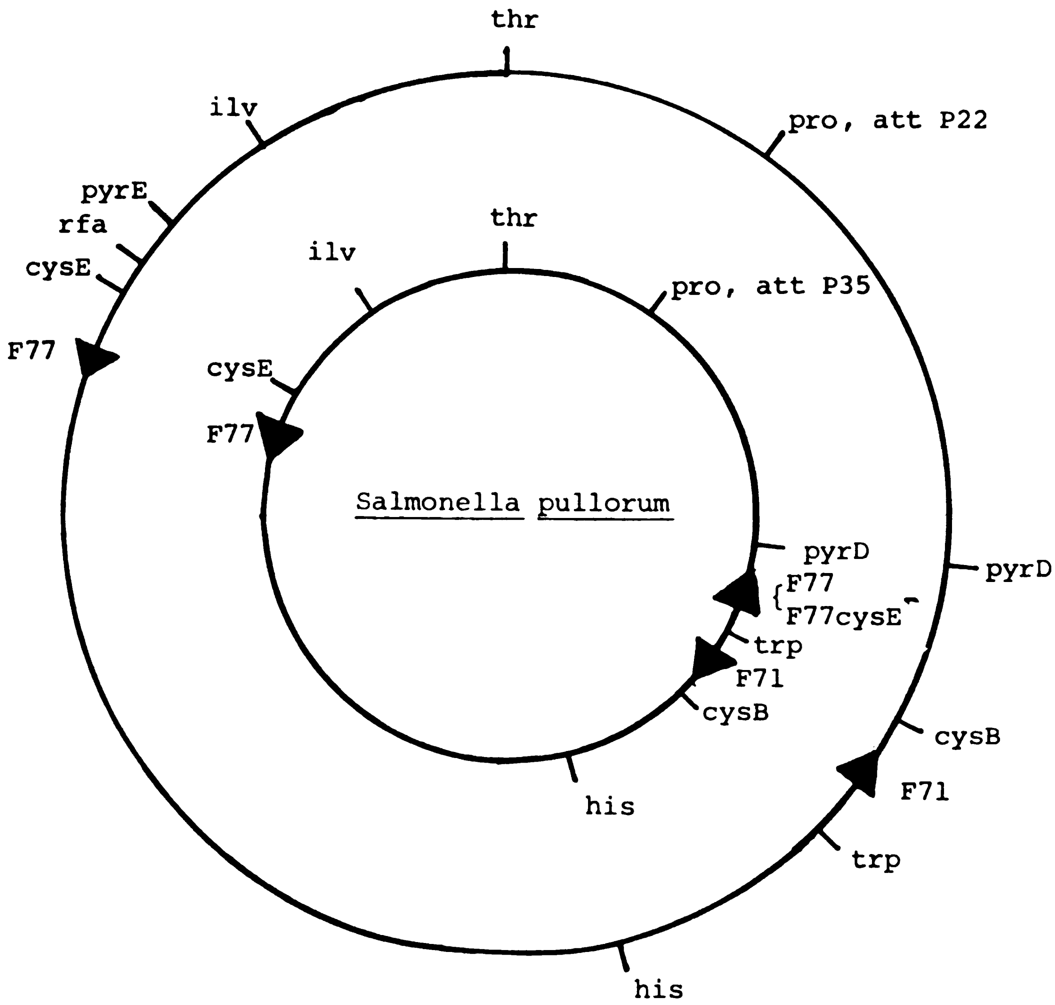
Part III

Spontaneous Mutation of F77

The donor MS831 carrying the F77cysE$^-$ transmissible plasmid appears to transfer the host chromosome from only one origin between the pyrD and trp genes in the clockwise direction (Table 7, Table 13, and Fig. 11) at a frequency slightly greater than MS830 when mated with MS104. Unlike F77, F77cysE$^-$ transfers ilv$^+$, thr$^+$ and pro$^+$ in a random, F$^+$ type of chromosomal transfer.

The absence of CysE$^+$ recombinants from MS831 x MS90 and MS831 x MS92 matings indicates that the F77cysE$^-$ mutation is either identical to the chromosomal cysE1 mutation or very near it or an overlapping deletion. The fate of the rfa$^-$ pyrE$^+$ portion is not known. The homology between F77cysE$^-$ and the chromosome may be due to a chromosomal sfa locus and the F portion of F77cysE$^-$ or to limited chromosomal nucleotide sequences and the cysE (rfa$^-$ pyrE$^+$) material of F77cysE$^-$ in the region between pyrD and trp in S. pullorum (Fig. 22). In S. typhimurium, there are two origins of transfer in the corresponding region between pyrD and trp (83).

Figure 22. The partial linkage maps of *S. pullorum* and *S. typhimurium*. The origins of transfer for F71, F77 and F77*cysE*$^-$ are shown as arrows.



Salmonella typhimurium

Figure 22

Part IV

Determination of a Homogeneous or Heterogeneous Donor Population Carrying F77 and Selection for Donors with Increased Fertility

Poisson distribution test. A derivative of MS830, designated MS832, was isolated from the Poisson distribution test and is a homogeneous population with regard to the cells carrying only one type of transmissible plasmid, namely F77, which can transfer the *S. pullorum* chromosome from two different origins, both in the clockwise direction. There seems to be a preference for transfer from the origin near *cysE*. This suggests that there is either a gradient of control favoring mobilization at the *cysE* locus or that F77 might exist autonomously in a few cells as a subpopulation of the donor MS830 or MS832 as described for a strain of Hfr *E. coli* (61). This subpopulation might be responsible for transfer from the origin between *pyrD* and *trp*. The former conclusion is favored since no F77 closed circular DNA was isolated from MS830 (Fig. 18B).

Matings using the donor MS832. The gradient of transfer, linkage and kinetic data (Table 7, Table 14, and Fig. 12) from the MS832 x MS374 matings are similar to those observed with the MS830 x MS374 matings (Table 7, Table 8, and Fig. 4), and further substantiates the origin and orientation of F77 chromosomal transfer as O-*ilv*-*thr*-*pro*.

The gradient of transfer, linkage and kinetic data (Table 7, Table 15, and Fig. 13) from the MS832 x MS104 matings are also similar to those presented from the MS830 x MS104 matings (Table 7, Table 12, and Fig. 10) and MS831 x MS104 matings (Table 7, Table 13, and Fig. 11), and further substantiates existence of a second origin for F77 transfer, also in the clockwise direction.

Electron microscopy of S. pullorum strains. Even though it has been possible to determine the origin and orientation of F77 and F77*cysE*$^{-}$ mediated chromosomal transfer in *S. pullorum* by gradient of transfer, linkage analysis and kinetic studies, the overall recombination frequencies for the selected markers are 10 to 100 fold less than normally observed with *S. typhimurium* donor strains. Therefore, the recipient strain MS374 and donor strains MS830, MS831, and MS832 were scanned in the electron microscope to look for gross surface structures that may be implicated in inhibiting pair formation. The recipient MS374 (Fig. 14A) appears to be free of unusual surface structures. The donor strains (Fig. 14B) however, appear to have an average of 15 or more sex-pili per cell. This is in great excess of the normal 2 to 3 sex-pili per *E. coli* donor cell (27). The many copies of the sex-pili per cell can result from either derepression of sex-pili synthesis (45) or relaxed replication control (9) of the F77 or F77*cysE*$^{-}$ factor.

Figure 14C shows what may be an actual mating pair. Depressed sex-pili synthesis may result in the formation of many nonfunctional sex-pili which can interfere with effective pair formation and conjugation. Similarly, multiple copies of F77 or F77<u>cysE</u>$^-$ may be responsible for an increased number of sex-pili. Another possibility is that multiple copies of the F-prime factor may result in competition for the homologous region on the chromosome and interfere with chromosomal mobilization and transfer. From the sucrose density gradient work it was calculated that there are 1 to 2 copies of F77 or F77<u>cysE</u>$^-$ in MS8300 and MS831, respectively. This favors the conclusion that there is derepressed sex-pili synthesis in the donor strains.

Part V

Evidence of F77 Converting to a More Stable Association with the Host Chromosome

As mentioned in the Materials and Methods section, the <u>S</u>. <u>pullorum</u> donors carrying F77 were very stable with regard to donor ability as measured by MS2 sensitivity.

The data in Fig. 15 indicate that with time and constant selection for a better donor, there is less transfer of the intact F77, as measured by MS2 sensitivity of the CysE$^+$ recombinants in the matings of MS8300 x MS90. Concomitantly, there is an increase in the recombination frequency of a selected donor chromosomal marker, ilv$^+$. Since cysE$^+$ is a plasmid gene, the recombinants must arise from either a reciprocal crossover between F77 and the donor chromosome in the F-cysE region or a nicking of the chromosomally associated F77 between F and cysE. This decrease in intact F77 transfer and increase in a chromosomal transfer is analogous to the conversion of MS8300 from the Type I F-prime donor to MS830, a Type II F-prime donors (76, 84).

The S. pullorum MS830 donor strain also has characteristics of both Type I F$^+$ and Type II F$^+$ E. coli donors described by Curtiss and Renshaw (29). Mobilization at the cysE locus is like a Type I F$^+$ donor since the F77 factor is very stable and mobilizes with a specific orientation, but with an intermediate frequency. Mobilization at the pyrD-trp locus is like a Type II F$^+$ donor which transfers at a low frequency which may be due to integration of F77 into the host chromosome but cannot express itself as a typical Hfr.

Part VI

Isolation and Characterization of Plasmid DNA

The MS83 isolate of S. pullorum strain MS35 apparently contains two plasmids: PO-1 and PO-2 (Fig. 18A) which are similar in size, 17s and 65s respectively, to those found in strain MS53 (73). However, the PO-2 plasmid in MS35 is not excluded by P35 phage as it is in MS53 (W. L. Olsen and D. E. Schoenhard, Bacteriol. Proc., p. 46, 1971).

The fact that closed circular DNA molecules were isolated from MS8300 (Fig. 20A) and that the sedimentation profiles with these molecules had a shoulder on the leading edge of the more rapidly sedimenting material indicates to me that F77 is still in the autonomous state (Fig. 19A). Confirmation of this conclusion is provided by cosedimentation of differentially labeled DNA from _S. typhimurium_ SA532 and MS8300 (Fig. 20B). The broad peak of activity extending from fractions 4 to 9 with material from MS8300 probably is due to the fact that it is a composite of F77 and plasmid PO-2.

The MS830 mutant of MS8300 which transfers from two origins: at _cysE_ between _pyrD_ and _trp_, and with an intermediate frequency, was suspected of being an Hfr donor in which F77 no longer existed autonomously. This conclusion was supported since no closed circular DNA of F77 size was observed (Fig. 18B). The Hfr type is probably the result of a mutation(s) in some regulatory mechanism.

The correlated loss of the PO-2 plasmid was unexpected since the autonomous state of neither F77 nor plasmid PO-2 was eliminated in MS831 (Fig. 19A) or MS8300 (Fig. 19B). Since neither F77 nor plasmid PO-2 remain autonomous in MS830 this is not a case of plasmid incompatibility as described by Novick (68) and reported between ColB2 and R(f) (45). An explanation of this observation is that F77 and PO-2 have a common control for maintenance as independent replicons. If the maintenance mechanism is mutated, then either the plasmid must come under the control of some other replicon, e.g., the chromosome, or abort. Because the F77 factor possesses homology with the chromosome at the *cysE* locus and probably at the *rfa* and *pyrE* loci, it can integrate while the PO-2 lacking known homology is lost.

The MS831 mutant of MS8300 which is F77*cysE*$^-$ *rfa*$^?$ *pyrE*$^?$ was similar to its progenitor except that mobilization of the chromosome occurred only between *pyrD* and *trp*. Apparently the mutation in the F77 factor was severe enough to prevent proper pairing and integration at the *cysE* locus. Probably integration at the *cysE* locus is a function of this gene rather than genes of the F. Since the mutation in the *cysE* locus is very stable, it is possible that the *rfa* and *pyrE* loci are also affected. If so, then integration between *pyrD* and *trp* must be due to F homology. This may account for the low frequency of transfer from this site. Tests of this conclusion rest upon isolating Hfr donors from MS831, and other mutants of MS8300 with which complementation and recombination studies can be done.

Approximately 88% of the plasmid DNA of *S. pullorum* MS35 derivatives exists as plasmid PO-1 DNA corresponding to 170 copies per cell. The PO-2-like plasmid DNA is present in only 1 to 2 copies per cell. The PO-1 and PO-2-like plasmids are probably cryptic plasmids as they have no known host phenotypic expression, similar to the small molecular weight plasmid (1.4 x 10^6 daltons) found by Cozzarelli *et al.* (25) in *E. coli* strain 15. Olsen and Schoenhard (73) observed approximately 150 copies of the PO-1 plasmid and 1 to 2 copies of the PO-2 plasmid per cell of *S. pullorum* MS53.

I conclude that the F-prime factor F77 exists in *S. pullorum* as either an autonomous replicon like a Type I F-prime donor or stably associated with the chromosome like a Type II F-prime donor. In either case, it transfers the host chromosome from two different origins; at *cysE* and between *pyrD* and *trp*, both in the clockwise direction. When the F77 factor becomes stably associated with the host chromosome, the PO-2 plasmid is lost. The F77*cysE*$^-$ factor transfers only from the origin between *pyrD* and *trp*. It exists as an autonomous replicon and the plasmid PO-2 is not lost. This suggests that there is a common control mechanism for the autonomous replication of the F-prime and the PO-2 plasmid. A mutation of this control mechanism may force the F77 factor to either abort or be rescued by associating with the host chromosome due to its chromosomal homology. Since there are only 1 to 2 copies of F77 or F77*cysE*$^-$ in the autonomous state per donor cell, the numerous sex-pili per donor cell must result from derepressed sex-pili synthesis.

SUMMARY

The transmissible plasmid F77 carrying the *cysE*$^+$ *rfa*$^-$ *pyrE*$^+$ genes transfers the *S. typhimurium* chromosome from the chromosomal *cysE* locus at 116 min on the chromosome map and in the clockwise direction (Sanderson and Saeed, personal communication).

The conclusion based on the gradients of transfer, linkage analyses and kinetic studies from the matings with *S. pullorum* donors carrying F77 and *S. pullorum* recipients is that F77 transfers the *S. pullorum* chromosome from two origins, both in the clockwise direction. F77 transfers primarily from the origin at the *cysE* locus which maps in the same relative position, 116 min, as in *S. typhimurium* and secondarily from the origin between the *pyrD* and *trp* loci which is equivalent to the 45 min position in *S. typhimurium*. The *S. pullorum* genetic markers studied appear to be in the same relative position as in *S. typhimurium* except for the inversion of the *trp cysB* loci. Presumably transfer from the origin at *cysE* is due to the homology between the *cysE rfa pyrE* loci on both F77 and the host chromosome. Transfer from the origin between *pyrD* and *trp* may be due to either a *sfa*

locus for the F portion of F77 or a nucleotide sequence with sufficient homology for the *cysE*$^+$ *rfa*$^-$ *pyrE*$^+$ portion of F77. The observation with MS830 of increased donor-capability, reduction in F77 transfer per se and an increase in transfer of donor markers, leads to the conclusion that F77 is stably associated with the host chromosome. The conclusion based on the results of the Poisson distribution test is that F77 is a homogeneous transmissible plasmid in the host cell.

The spontaneous mutant of F77 designated F77*cysE*$^-$, transfers the *S. pullorum* chromosome only from the origin between *pyrD* and *trp* in the clockwise direction at the same frequency as F77. Transfer from this origin must be due to either a chromosomal *sfa* locus for the F portion of F77*cysE*$^-$ or a nucleotide sequence with sufficient nucleotide sequence homology for the chromosomal portion of F77*cysE*$^-$. The low frequency of transfer suggests that F77*cysE*$^-$ does not form a stable association with the chromosome.

Electron micrographs show that *S. pullorum* donors harboring F77 and F77*cysE*$^-$ have at least 15 sex-pili per cell. The recipient cell has no unusual gross cell surface structures. Many sex-pili appear to be involved in mating pair formation.

Supercoiled DNA molecules can be isolated from *S. pullorum* MS35 derivates and an *S. typhimurium* donor strain by CsCl-EtBr dye buoyant density gradient centrifugation and characterized by zonal centrifugation in a linear neutral sucrose gradient. Using these methods, it was found that the *S. pullorum* recipient MS83 has two distinct plasmid molecules designated PO-1 and PO-2. Plasmid PO-1 has a molecular weight of 2.1 x 10^6 daltons, sedimentation coefficient of 17s and present in about 170 copies per host chromosome. The PO-2 plasmid is not excluded by phage P35, and has a molecular weight of 45 x 10^6 daltons, sedimentation coefficient of 65s and present in about 1-2 copies per host chromosome. The donor MS830 carrying F77 has only the PO-1 plasmid molecules present. The PO-2 plasmid appears to be excluded by F77. When the recipient MS83 is newly infected with F77 (MS8300), the PO-2-like plasmid peak appears broader and is a combination of two plasmid species, a 65s and 70s molecule. The donor MS831 carrying F77*cysE*$^-$ has the PO-1 plasmid species and the broad PO-2-like plasmid species which also is a combination of two plasmid species, a 65s and 70s molecule.

The plasmid molecule isolated from the *S. typhimurium* donor SA532 carrying F77 appears to be homogeneous and co-sediments with the 70s plasmid molecule of the broad PO-2-like peak of MS8300 and MS831. Further studies indicate that F77 has a molecular weight of 51 x 10^6 daltons and sedimentation coefficient of 70s. This suggests that F77 is autonomous in *S. pullorum*, but when it becomes associated with the host chromosome, the PO-2 plasmid molecule is lost. This suggests that the PO-2 plasmid and F77 factor may have a common replication control mechanism. A spontaneous mutation may have inhibited replication, but F77 can associate with the chromosome due to homology and be maintained. The lack of PO-2 plasmid exclusion by F77*cysE*$^-$ suggests that the spontaneous mutation resulting in the mutation of the F77*cysE*$^+$ gene has not disrupted PO-2 and F77 plasmid replication control.

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