A CONJUGATION SYSTEM IN SALMONELLA PULLORUM

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Mary Judith Robinson
1964

This is to certify that the

thesis entitled

A Conjugation System in
Salmonella pullorum

presented by

Mary Judith Robinson

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology & Public Health

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Date September 3, 1964

ABSTRACT

A CONJUGATION SYSTEM IN SALMONELLA PULLORUM

by Mary Judith Robinson

A conjugation system was established in Salmonella pullorum by the identification of a naturally occurring high frequency recombination (Hfr) strain, S. pullorum 6, and by the introduction of F^+ and $F'lac^+$ sex factors into S. pullorum 35 followed by the isolation of Hfr mutants of these cultures. The maleness of F^+, F', and Hfr strains of S. pullorum was determined by a positive staining reaction, ability to transfer the staining reaction, ability to be cured of the staining reaction, ability to conduct chromosomal markers to recipient strains, and insusceptibility to a female specific S. pullorum bacteriophage.

S. pullorum 35 was shown both cytologically and genetically to be a recipient population. Electron microscopic examination of mating mixtures demonstrated the ability of S. pullorum 35 to form effective conjugal pairs with and to receive genetic material from an E. coli donor. Genetic evidence indicated that strain 35 is an F^- rather than F^0 recipient.

Two Hfr strains of S. pullorum with different origins and opposite orders of entry of genetic characters were used to determine the positions of several markers on the donor chromosome. Three mapping procedures--gradient of transmission, genetic constitution of recombinants, and interrupted mating--were employed. The order of markers on the S. pullorum donor linkage group was found to be his--(pro)--leu--(ara)--mot--cys--(ile)--mtl--gal--str--xyl. The S. pullorum

chromosome was shown to be a closed continuous structure which becomes discontinuous and linear during transfer from an Hfr strain.

S. pullorum Hfr-1 behaved classically with regard to the conduction of genetic determinants to recipient cells. Both Hfr-1 and S. pullorum 6 formed unusually stable effective pairs with an S. pullorum recipient.

A CONJUGATION SYSTEM IN SALMONELLA PULLORUM

By

Mary Judith Robinson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1964

This thesis is dedicated to
my parents who gave me an intellect
and to
the educators who developed it.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. Delbert E. Schoenhard for his patience, encouragement, and guidance throughout the course of this study and especially for his continued interest and the constant challenge his suggestions presented.

I should also like to acknowledge the aid and constructive criticism of my fellow graduate students.

A special note of thanks is due to Mr. S. S. Tevethia for his valuable advice and assistance.

During the course of this study, I was supported in part by a National Science Foundation Summer Fellowship for Graduate Teaching Assistants.

M. J. R.

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INTRODUCTION

The discovery of bacterial conjugation in Escherichia coli K-12 was perhaps the most important event in the recent history of bacterial genetics. The importance of this discovery lies in the fact that conjugation permits a rapid genetic analysis of the entire bacterial chromosome. Genetic analyses by conjugation have led to the pictorial representation of the bacterial chromosome as a circle each point of which represents a single mutation. The interval between two points signifies a quantitative expression of the amount of DNA separating two mutant sites. The existence of a circular linkage group of linearly-ordered genetic determinants is well established in E. coli and may be true of bacteria in general.

Genes responsible for the synthesis of cellular metabolites and genes responsible for the utilization of exogenous energy sources have been shown to be arranged on the bacterial chromosome in two fashions. Most commonly genes involved in a specific pathway are located within a limited region of the chromosome in a coterminus arrangement. For several biosynthetic pathways a linkage of biochemically related genes is not observed. The eight genes controlling the formation of enzymes required for arginine synthesis, for instance, occur in one cluster of four genes with the remaining four genes distributed singly in widely separated areas of the E. coli and Salmonella typhimurium chromosomes.

Evidence has accumulated which suggests that a correlation exists between virulence and the ability to synthesize arginine in Salmonella pullorum. Because of the scattering of arginine loci on the bacterial

chromosome and because bacterial conjugation permits a rapid gross structure analysis of the entire chromosome, conjugational analysis of the S. pullorum linkage group represents a rational approach to the future genetic study of virulence in this organism.

The following study was undertaken to establish a conjugation system in S. pullorum and to genetically analyze the S. pullorum linkage group. This purpose was to be accomplished by the identification of a natural conjugation system or by the introduction of sex factors and subsequent isolation of mating types followed by recombinational studies on the S. pullorum chromosome.

LITERATURE REVIEW

Review of the Conjugation Process

Bacterial conjugation is most accurately and simply described as a unidirectional transfer of genetic material from one bacterial cell to another where the transfer requires contact between cells (Clark and Adelberg, 1962).

Mating Types of Bacteria--Cultures of Escherichia coli capable of conjugating, or mating, fall into two classes: those which donate genetic material and those which receive genetic material. By anology to higher organisms the donor cultures are called male and the recipient cultures are called female. Male cultures owe their ability to donate to genetic elements known alternately as sex factors, fertility factors, or F-factors and are thus referred to as F^+ (Jacob and Wollman, 1961). Female cultures which lack fertility factors but which possess chromosomal markers enabling them to mate with male cells (Johnson, et al., 1964) are called F^-.

Four criteria distinguish a male from a female culture. (1) The surface properties of donor cells are altered so as to allow male bacteria to pair specifically with F^- recipients (Jacob and Wollman, 1961). The difference in surface structure between donor and recipient bacteria is revealed by: (a) the ability of a male cell to form effective pairs with female cells, (b) the altered antigenicity of a donor cell (Le Minor and Le Minor, 1956; Ørskov, I. and F. Ørskov, 1960), (c) the sensitivity of male cultures to male specific RNA containing bacteriophages (Loeb, 1960; Loeb and Zinder, 1961) and conversely their resistance to female specific bacteriophages,

(d) the permeability of a male cell to the dye eosin (Zinder, 1960b). (2) Donor cultures transfer promotor genes to recipient cells (Jacob and Wollman, 1961). (3) Donor cultures conduct chromosomal markers to recipient cells (Hayes, 1964). (4) All male characteristics may be eliminated from a donor population by treatment with acridine dyes (curing) (Hirota, 1960).

In Salmonella typhimurium a third class, the neuter or F^0 class, has been described (Baron, et al., 1959). This class of bacteria is capable of neither donating nor receiving genetic information by means of conjugation.

Alteration of Mating Type--The mating type of a cell may be altered in several ways. A male cell may be converted into a female cell by loss of the F-factor either as a result of spontaneous mutation (Jacob et al., 1960) or experimental removal, resulting from treatment with acridine dyes (Hirota, 1960). An F^0 cell may become F^+ by spontaneous mutation (Baron et al., 1959). An F^- cell may be converted to an F^+ type only by receiving an F-factor from a male culture (Jacob and Wollman, 1961).

Classification of Fertility Factors--Fertility factors may be divided into two classes: (1) F-factors containing promotor genes only and (2) F-factors containing promotor genes plus one or more chromosomal genes attached to and transferred simultaneously with the promotor. Promotor genes are those genetic determinants responsible for the formation of effective conjugal pairs by alteration of the male cell surface, mobilization of the donor genetic material, and provision of an immediate energy source required for genetic transfer (Clark and Adelberg, 1962).

The best known sex factor of the first type is the F-agent of E. coli K-12 (Cavalli et al., 1953). An example of the second class

of sex factors is the F' or sexducing (also called F-ducing or F-merogenote) of E. coli (Jacob et al., 1960). The size of the chromosomal segment which can be incorporated into a sex factor is variable. It is, however, generally small, consisting of only those genes associated with a single operon (Jacob and Wollman, 1961). The chormosomal region of an F-particle may, on the other hand, represent as much as one-tenth of the bacterial linkage group and may include genes responsible for as many as four separate biosynthetic pathways in E. coli (Pittard and Adelberg, 1964).

Each class of F-elements is divided into two subgroups: (a) episomes and (b) plasmids. Episomes are genetic elements capable of existing autonomously in the cytoplasm where they replicate independently of and more rapidly than the bacterial chromosome (Lederberg et al., 1952; Cavalli et al., 1953) and of becoming integrated into the bacterial linkage group (Jacob and Wollman, 1961). When integrated the F-factor replicates in synchrony with the bacterial genome. In the autonomous state F-episomes are donated independently of chromosomal transfer to recipient cells at a high frequency. When an F-episome is integrated into the bacterial chromosome the entire chromosome may be transferred to recipient cells during conjugation. A mutant of an F^+ culture containing integrated promotor genes is referred to as a high frequency recombination (Hfr) mutant, since, once isolated, this mutant culture will transfer chromosomal markers to recipient cells at a high frequency.

An F-episome may become stably attached to the chromosome at any site. At whatever point the F-particle interacts with the chromosome the linkage group becomes linear (Clark and Adelberg, 1962). The linear chromosome is then transferable to recipient cells in an oriented and sequential manner such that the point of interaction, the

origin, enters first followed sequentially by chromosomal markers with the integrated sex factor (Hfr gene) entering last (Jacob and Wollman, 1961).

An F' episome differs from an F-episome in its ability to integrate with the chromosome. The F' element is obligated to enter the chromosome at the site of whatever chromosomal gene it carries (Adelberg and Burns, 1959). The obligatory point of entrance is presumably the result of a necessity of base pairing homology between the chromosomal marker episome and the chromosome. The result of this directed integration site is an Hfr strain with a predictable order of entry according to which the attached exogenotic marker enters late followed only by the terminal Hfr gene.

Three criteria distinguish an Hfr from an F^+ donor. (1) An Hfr strain transfers genetic markers at a frequency at least one thousand fold greater than the F^+ culture from which it was derived. (2) The transfer of markers occurs in an oriented manner such that every recipient cell mating with an Hfr donor receives the donor genetic characters in the same order. The transfer of markers in an F^+ culture, on the other hand, is random as a consequence of the presence of several Hfr mutants with differing orders of entry in the culture. (3) The promotor is donated at a low frequency in Hfr cultures rather than at the high frequency characteristic of F^+ cultures.

Plasmids, unlike episomes, are capable of existing solely in the autonomous state in the bacterial cytoplasm (Lederberg, 1952). Plasmids are donated to recipient cells at a high frequency, usually in the absence of chromosomal transfer (Clark and Adelberg, 1962).

Modification of Fertility Factors--Fertility factors may be modified in three ways; (1) by mutational loss of a promotor function; (2) by recombination with the bacterial chromosome; or (3) by alteration of the physiological state of the donor.

Ultraviolet treatment of strains of E. coli possessing fertility factors produces cells which are male in all respects with the exception that they are no longer capable of transferring genetic material to recipient cells (Jacob and Wollman, 1961). Sex factors of this type have lost at least one promotor function and are referred to as mutant promotors. Cells containing modified F-particles are, in addition, incapable of receiving genetic material from known donor strains (superinfection) by means of conjugation (Mäkelä et al., 1962). Such a mutant promotor may be responsible for the F^0 nature of S. typhimurium cultures (Clark and Adelberg, 1962).

During growth of Lac^-/Lac^+ F' heterogenotes, cells which exhibit the lac^- phenotype of the parent recipient segregate. The genotypes of such segregants may be determined by genetic tests. A minority are haploid parentals. The majority, however, still carry an F'lac factor which, as a result of mitotic recombination carries the chromosomal lac^- mutation (Jacob and Wollman, 1961). In such cases it is the attached chromosomal segment rather than the promotor itself which is altered.

The physiological condition of the donor cell may also prevent expression of a promotor function. When mated in the stationary phase of growth, aerated, or when treated with periodate all cells in a male population phenocopy F^- (cf. Clark and Adelberg, 1962). The original male phenotype is restored when the restrictive physiological conditions are removed and the cells are grown through one or two generations (Hayes, 1964).

Successive Steps in the Conjugation Process--The conjugation process consists of four distinct steps: (1) formation of effective conjugal pairs; (2) conduction or donation of genetic material from the donor to the recipient cell; (3) expression of newly received genes; (4) formation of a stable recombinant cell.

The formation of a conjugal pair procedes in three stages: (1) cells of opposite mating type collide and undergo an unstable attachment (contact); (2) the cell boundaries separating the two cells disappear in the area of contact; (3) a stable cytoplasmic bridge connecting the cells is formed (Jacob and Wollman, 1961).

Contact between cells in a mating mixture is a random process depending solely upon chance collision. No attracting substance is produced by either donor or recipient cells (Jacob and Wollman, 1961). Thus, donor cells may collide with other donor cells as well as with recipient cells. Two coupled cells of opposite mating type are referred to as a specific pair (Clark and Adelberg, 1962). A specific pair is converted into an effective conjugal pair when all the conditions necessary for genetic transfer--i.e., formation of a cytoplasmic bridge, preparation of the genetic material for transfer (mobilization), and provision of an immediate energy source for the process of transfer--have been achieved (Clark and Adelberg, 1962).

Once the appropriate conditions have been established, donor genetic material is transferred to recipient cells. Two types of transfer are distinguished. In one case the promotor is transferred independently of the chromosome. In the second case the promotor and chromosomal markers are transferred as linked elements. The term conduction has been envoked to describe this second type of transfer (Clark and Adelberg, 1962).

Transfer of genetic material is followed by expression of the newly received DNA immediately (Riley et al., 1960) or within several generations. The time required for expression depends upon the complexity of the introduced character (Jacob and Wollman, 1961).

The events which occur between the time of expression of transferred genes and the formation of a stable recombinant cell are unknown. Conjugational analyses have been of little help in the elucidation of the

mechanism of recombinant formation in exconjugate cells (Clark and Adelberg, 1962).

Frequency of Genetic Transfer during Conjugation--The frequency with which chromosomal markers are transferred to recipient cells during conjugation depends upon (1) the location of the F-factor in the donor cell and (2) the recipient capacity of the female culture.

Male cultures with F-episomes primarily in the autonomous state transfer chromosomal markers to F^- cells at a low frequency as measured by the number of recombinant cells formed. The frequency of recombinants formed in low frequency recombination crosses ($F^+ \times F^-$) does not exceed one recombinant cell per 10^5 donor cells (Jacob and Wollman, 1961). The formation of these recombinants is due to the occurrence of rare Hfr mutants in the F^+ population.

Male cultures containing integrated sex factors (Hfr strains) transfer chromosomal markers to F^- cells at a high frequency. The frequency of chromosomal marker transfer in high frequency recombination crosses (Hfr x F^-) is at least 1,000 fold greater than that observed in an $F^+ \times F^-$ cross (Jacob and Wollman, 1961).

Both types of male cultures transfer chromosomal markers to an F^+ culture at a frequency about one-tenth as great as that observed when F^- cells are used as recipients (Jacob and Wollman, 1961). The ability of two male cultures to interconjugate is dependent upon the occurrence of spontaneous Hfr and F^- mutants in both cultures.

Similarly, the frequency with which an F-factor is transferred to recipient cells during conjugation depends upon the state of the promotor. Cytoplasmic F-factors are transferred with an efficiency approaching 100%. In E. coli 70% of the recipient cells receive the sex factor within five minutes of the onset of conjugation (Jacob and Wollman, 1961).

An integrated F-factor is transferred at a frequency of approximately one thousandth of the frequency of the proximal marker (Jacob and Wollman, 1961). The low frequency of transmission of an integrated F-particle is a reflection of (1) its terminal position on the linear chromosome and (2) the probability of random chromosomal rupture during conduction to recipient cells.

The frequency with which a specific marker is conducted to a recipient cell depends upon (1) its distance from the origin on the chromosome, (2) the rate of chromosomal transfer, and (3) the resistance of specific pairs to spontaneous and operational disruption.

When a population of Hfr cells is allowed to mate with F^- cells undisturbed for a suitable length of time and is then plated on media selective for recombinants having received different markers from the Hfr, a gradient of transmission of genetic material is observed (Hayes, 1964). This gradient results from spontaneous rupture of the chromosome during transfer. Assuming that the probability of rupture is constant per unit length of the chromosome and that the rate of transfer of the chromosome from donor to recipient is constant, the observed recombination rate for a given Hfr marker is the negative exponential function of that marker's distance (measured in time units) from the point of origin (Jacob and Wollman, 1961). The direct relation between the distance from the origin and the recombination frequency of an Hfr marker has been experimentally confirmed by Taylor and Adelberg (1960).

The genetic polarity of the Hfr chromosome allows a distinction between two categories of genetic characters: those for which the frequency of recombination is high and those for which it is low (Hayes, 1953). That portion of the Hfr chromosome carrying genes of the first category is called the proximal segment. That portion of the Hfr chromosome carrying genes of the second category is called the distal segment. The existence of genetic polarity in Hfr strains indicates that

the Hfr chromosome is a linear, discontinuous structure which is defined by two extreminities: the origin and the Hfr gene (Jacob and Wollman, 1961).

The order in which donor markers are transferred is characteristic and constant for the Hfr strain employed. By varying the means of selection, Hfr strains may be isolated which differ with respect to the characters they transfer with high frequency. Whereas one Hfr strain may transfer two markers as if they were located distal to one another on the Hfr chromosome, a second Hfr isolate may transfer the same two markers with a frequency characteristic of proximal markers. These anomolies of transfer are reconciled by picturing the F^- and F^+ chromosome as a closed, continuous structure which is transformed into a linear, discontinuous, transferrable form during mating by random interaction with an F-factor (Jacob and Wollman, 1958; 1961).

Genetic Analysis of a Linkage Group by Conjugation

Three methods of genetic analysis have been used to dissect the bacterial chromosome by means of conjugation. These methods and their applications are described briefly below. A more detailed evaluation is presented in Material and Methods.

Mapping by the Gradient of Transmission--Genetic characters of an Hfr strain are transmitted to recombinants with different and characteristic frequencies. The genetic constitution of zygotes depends solely on random chromosomal breakage during transfer which is, in turn, dependent upon the distance of the markers from the origin of the Hfr chromosome. The genetic characters of the Hfr strain thus may be ordered according to the decreasing frequency of their transmission (Jacob and Wollman, 1961). Since chromosomal breakage is random,

however, this method is also valid for estimating the distances between markers as well as their relative order (Hayes, 1964).

Mapping by Genetic Analysis of Recombinants--Using a few appropriate selections, linkage relationships may be established between unselected Hfr markers and those characters chosen for the selection of recombinants. This method is suitable for mapping genetic characters distal to the selected markers (Jacob and Wollman, 1961).

Mapping in Time Units--Genes on the proximal segment of the donor chromosome may be ordered in relation to the leading extremity by determining the specific time at which each marker begins to appear in recombinants following separation of mating pairs at specified time intervals by means of violent agitation (Hayes, 1964; Jacob and Wollman, 1961).

Pictorial Representation of the E. coli and S. typhimurium Chromosomes

The chromosomes of both E. coli and S. typhimurium have been extensively mapped by conjugational analyses. The circular linkage groups of E. coli (Hayes, 1964; Jacob and Wollman, 1961) and S. typhimurium (Sanderson and Demerec, 1964) are presented in Fig. 1 and 2. Figure 3 indicates regions of homology on the two chromosomes.

Occurrence of Intra- and Intergeneric Conjugation

Intrageneric conjugation has been observed in the following genera of bacteria: Escherichia (Lederberg and Tatum, 1946), Salmonella (Zinder, 1960b), Pseudomonas (Holloway and Fargie, 1960), and Serratia (Belser and Bunting, 1956).

Intergeneric conjugation has been reported to occur between the following pairs of genera: Escherichia-Serratia; Salmonella-Serratia;

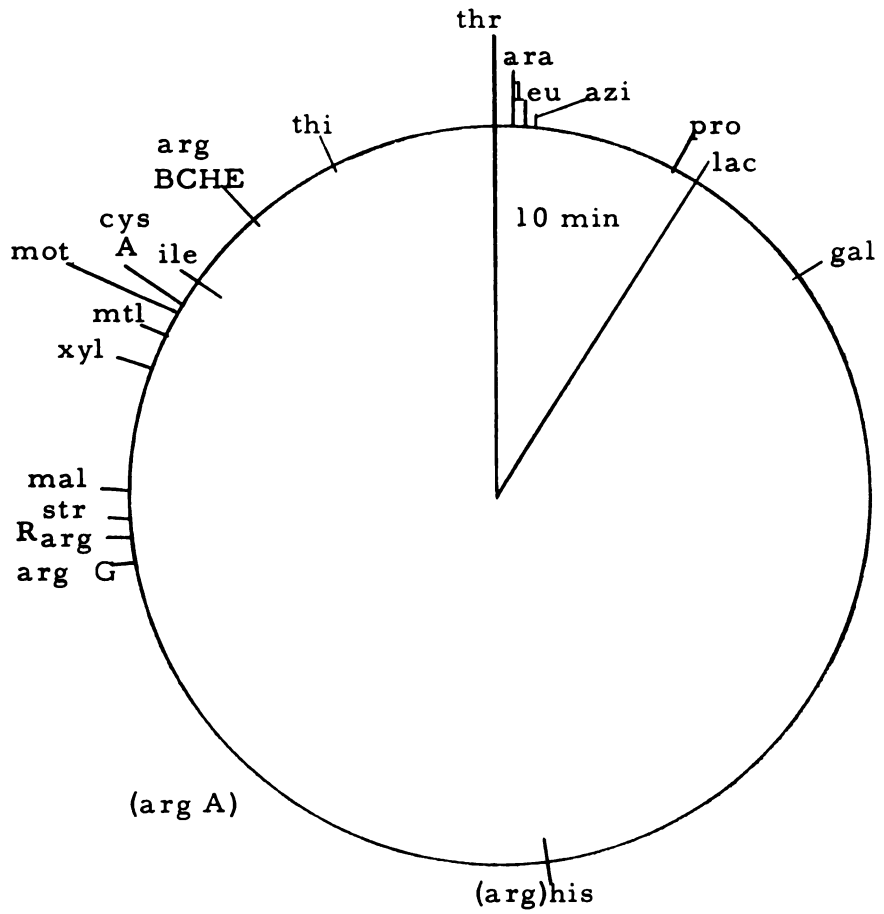

Figure 1. Positions of some markers on the circular E. coli linkage group.

Abbreviations: thr=threonine; ara=arabinose; leu=leucine; azi=azide; pro=proline; lac=lactose; gal=galactose; his=histidine; arg=arginine; R=regulator; str=streptomycin; mal=maltose; xyl=xylose; mtl=mannitol; mot=motility; cys=cysteine; ile=isoleucine; thi=thiamine. Markers whose exact position is uncertain appear in parentheses.

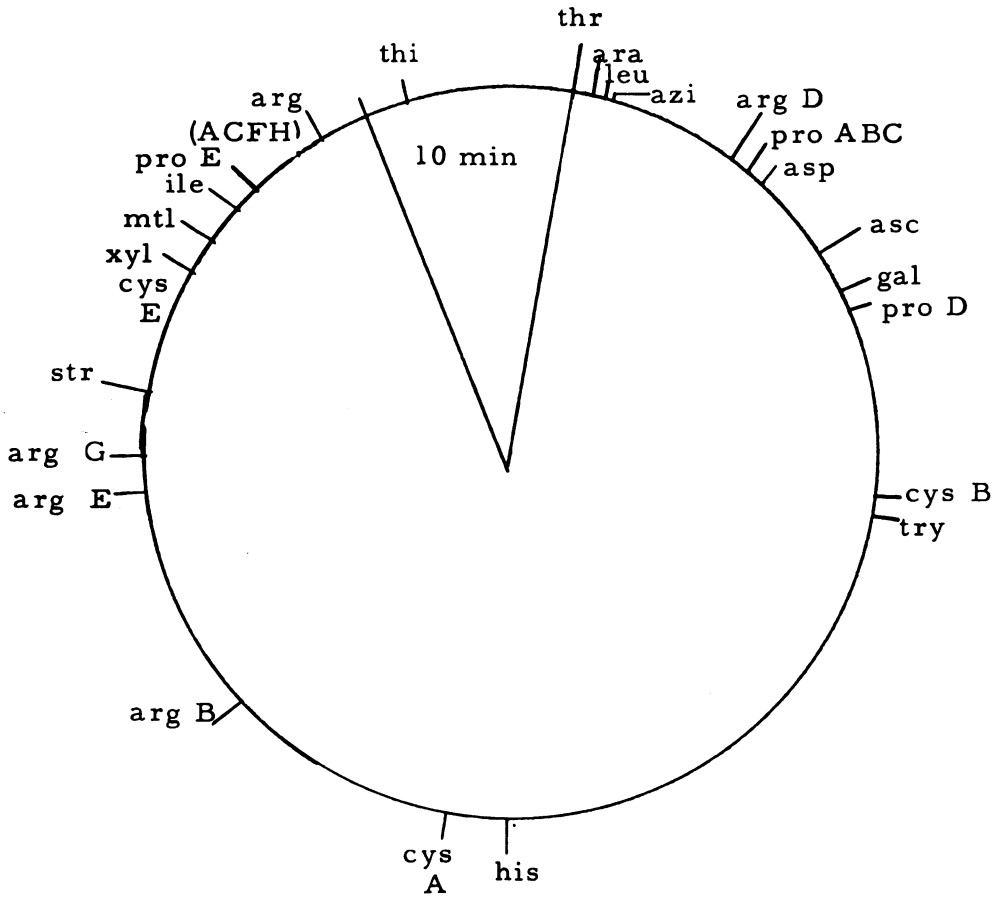

Figure 2. Positions of several markers on the circular S. typhimurium linkage group.

Abbreviations: thr=threonine; ara=arabinose; leu=leucine; azi=azide; arg=arginine; pro=proline; asp=aspartic acid; asc=ascorbic acid; gal=galactose; cys=cysteine; try=tryptophane; his=histidine; str=streptomycin; xyl=xylose; mtl=mannitol; ile=isoleucine; thi=thiamine.

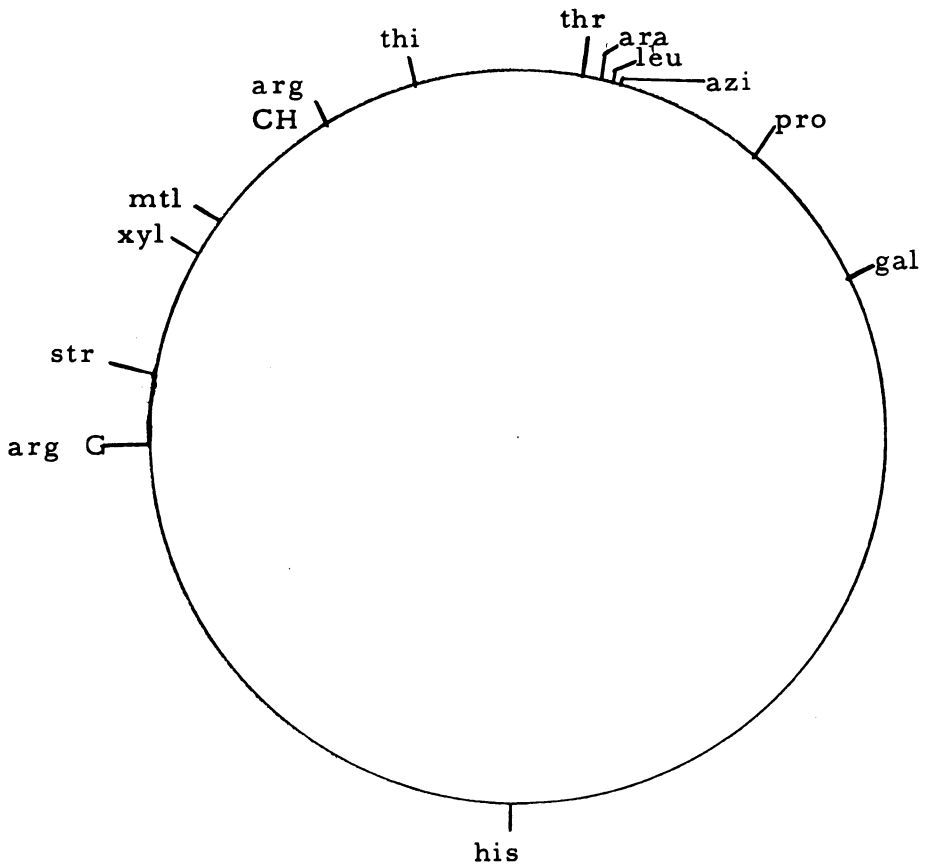

Figure 3. Regions of homology between the E. coli and S. typhimurium chromosomes.

Abbreviations: thr=threonine; ara=arabinose; leu=leucine; azi=azide; pro=proline; gal=galactose; his=histidine; arg=arginine; str=streptomycin; xyl=xylose; mtl=mannitol; thi=thiamine.

Shigella-Salmonella; Escherichia-Salmonella (cf. Clark and Adelberg, 1962); and Escherichia-Proteus (Falkow et al., 1964). Conjugation between Escherichia and Salmonella has been shown to occur in a mammalian host (Falkow and Baron, 1962). In most of these cases, however, evidence that the observed genetic transfer requires or even involves cell contact is incomplete (Clark and Adelberg, 1962; Miyake, 1962).

MATERIALS AND METHODS

Cultures

The bacterial strains employed and the genetic characteristics of each which are pertinent to this study are listed in Table I. Various mutant markers and sex factors were added to Salmonella pullorum 35W as required. These mutants as they appear in the text are designated 35 followed by the mutant genotype. Forty-seven additional strains of S. pullorum were employed to survey for donor ability. The strains surveyed included S. pullorum 1-38, 43, and 45-54.

Nineteen bacteriophages isolated from lysogenic strains of S. pullorum (Robinson, unpublished) were used to study promotor induced alterations of the male cell surface. These bacteriophages are designated: 1, 4, 5, 6, 12, 15, 16, 22, 24, 26, 29, 33, 34, 36, 37, 38, 40, 46, and 50.

Media

A list of the media used and the purpose of each is given in Table II.

Composition of Synthetic Media

Minimal E Medium (Vogel and Bonner, 1956)

$MgSO_4 . 7H_2O$	0.2 g
Citric acid. H_2O	2.0 g
K_2HPO_4 anhydrous	10.0 g
$NaHNH_4PO_4 . 4H_2O$	3.5 g
Carbohydrate	4.0 g
Distilled water	1000 ml

TABLE I.--Characteristics of bacterial strains *.

Strain	Mating type	Auxotrophic characters	Carbohydrate utilization					Ind	Mot	Response to str
			lac	gal	ara	xyl	mtl			
Salmonella pullorum 35W	F^-	$cys^- leu^-$	-	+	+	+	+	-	-	s
6	(F+)	$cys^- leu^- pro^-$	-	+	+	+	+	-	-	s
Escherichia coli W6	F+	met^-	+	+	+	+	+	+	+	s
104	F^-	$thr^- leu^- thi^-$	-	+	+	+	+	+	+	s
AB266	F^-	$thr^- leu^- thi^- pro^-$	-	-	-	-	-	+	+	r
AB113	F^-	$thr^+ leu^+ thi^+ his^-$	-	-	+	-	-	+	+	r
107	F^-	$thr^- leu^- thi^-$	-	-	-			+	+	s
AB785	F'Lac	thi^-	+	+	+	+	+	+	+	s

*Abbreviations and symbols: cys=cysteine; pro=proline; leu=leucine; met=methionine; thr=threonine; thi=thiamine; his=histidine; lac=lactose; gal=galactose; ara=arabinose; xyl=xylose; mtl=mannitol; +=utilized or produced; - =not utilized or produced; ind=indole production; mot=motility; s=sensitive; r=resistant.

Table II.--A list of media used and the purpose of each.

Medium	Source	Purpose	Supplements
Penassay broth	Difco	growth medium	
Levine's EMB Without lactose (EMO)	Baltimore Biological Laboratory	differential medium: carbohydrate utilization; male cultures	0.8% casamino acids 0.4% carbohydrate (omitted for detection of male cultures)
Levine's EMB Agar (EMB-lac)	Difco	differential medium: lactose utilization	0.8% casamino acids
Phenol red broth Base (PR)	Difco	differential medium: carbohydrate utilization	0.4% carbohydrate 1.5% Bacto-Agar (Difco) or 0.7% Bacto-Agar (Difco) (soft agar)
SIM	Difco	differential medium; motility, indole and H_2S production	
Methylene blue-Bromcresol blue Agar (MB-BCP)	Difco	differential medium: lactose utilization; selective medium: E. coli	
Brilliant green Agar	Difco	differential medium: lactose utilization; selective medium: Salmonella	

Minimal E medium	selective medium: detection of recombinants	see Table IV for supplements added to E medium for specific selections
Double enriched E Medium	differential medium: amino acid and nucleotide mutants	E medium plus 0.25% v/v nutrient broth
Pool media 1-9	identification of amino acid and nucleotide mutants	E medium plus pools 1-9
Tryptone agar	growth medium for detection of phage lysis	
M-9 broth	growth medium	
Watanabe (W) medium	Selective medium: detection of recombinants	see Table IV for supplements added to W medium for specific selections

Minimal E. Agar--Double strength agar (15 g Bacto-Agar per 500 ml distilled water) and double strength E salts were autoclaved separately and mixed when both solutions had cooled to 45C.

Pool Media--Nine pool media were prepared by supplementing minimal E agar with 10 ml/liter of amino acid and purine and pyrimidine pools 1-9. The composition of pools 1-9 is given in Table III.

Watanabe (W) Medium (Watanabe and Watanabe, 1959).

K_2HPO_4	0.3 g
KH_2PO_4	0.1 g
NH_4Cl	0.5 g
Na_2SO_4	0.1 g
$MgSO_4$	0.2 g
Carbohydrate	0.5 g
Distilled water	1000 ml

Soft Agar

Bacto-Agar	7.0 g
Distilled water	1000 ml

Composition of Complex Media

Tryptone Agar

Bacto-Tryptone	10 g
Bacto-Agar	10 g
NaCl	8 g
Dextrose	5 g
N $CaCl_2$	0.1 ml
Distilled water	1000 ml

TABLE III.--Composition of amino acid and purine and pyrimidine pools* [1]

Pool no.	1	2	3	4	5
6	adenine	guanine	thymine	methionine	thiamine
7	histidine	uracil	isoleucine	valine	lysine
8	phenyl-analine	tyrosine	tryptophane	threonine	proline
9	glutamic acid	glycine	alanine	aspartic acid	arginine

*After Hartman et al., 1962

[1] Concentrations of supplements: amino acids and purines and pyrimidines = 2 mg/ml; vitamins = 0.5 mg/ml.

TABLE IV.--Supplements added to E or W medium for specific selections*+1

Medium number	Dex	Gal	Xyl	Mtl	Thi	His	Cys	Thr	Leu	Ile	Pro	Str	Azi	Markers selected
1	+	-	-	-	+	-	+	+	+	-	-	+	-	$his^{+}str^{r}$
2	+	-	-	-	-	+	+	-	+	-	-	+	-	$thr^{+}thi^{+}str^{r}$
3	-	+	-	-	+	+	+	+	+	-	-	+	-	$gal^{+}str^{r}$
4	-	-	+	-	+	+	+	+	+	-	-	+	-	$xyl^{+}str^{r}$
5	-	-	-	+	+	+	+	+	+	-	-	+	-	$mtl^{+}str^{r}$
6	-	+	-	-	+	+	+	+	+	-	-	-	-	gal^{+} #
7	-	-	+	-	+	+	+	+	+	-	-	-	-	xyl^{+} #
8	-	-	-	+	+	+	+	+	+	-	-	-	-	mtl^{+} #
9	+	-	-	-	+	-	-	+	+	-	-	+	-	$his^{+}cys^{+}str^{r}$
10	+	-	-	-	-	-	+	-	+	+	+	+	-	$his^{+}str^{r}$
11	+	-	-	-	-	+	+	-	+	-	+	+	-	$ile^{+}str^{r}$
12	+	-	-	-	-	+	+	-	+	+	-	+	-	$pro^{+}str^{r}$

13	+	-	-	-	-	-	+	-	+	-	+	+	-	$thr^{+}thi^{+}str^{r}$
14	+	-	-	-	-	-	+	-	+	+	+	-	+	$his^{+}azi^{r}$
15	+	-	-	-	-	+	+	-	+	-	+	-	+	$ile^{+}azi^{r}$
16	+	-	-	-	-	+	+	-	+	+	-	-	+	$pro^{+}azi^{r}$

*Abbreviations and symbols: dex=dextrose; gal=galactose; xyl=xylose; mtl=mannitol; thi=thiamine; his=histidine; cys=cysteine; thr=threonine; leu=leucine; ile=isoleucine; pro=proline; str=streptomycin; azi=azide; r=resistence; s=sensitive; +=added; - = not added; for markers selected: += utilized or produced; - = not utilized or produced.

+Concentrations of supplements: amino acid supplements were added to give a final concentration of 20μg/ml; vitamins 5μg/ml; carbohydrates 0.4%; streptomycin sulfate 500μg/ml; sodium azide 0.032%.

[1] Amino acid and vitamin supplements and sodium azide were added to double strength E salts prior to autoclaving. Streptomycin sulfate and carbohydrates were added after autoclaving when the medium had cooled to 45C.

#In S. pullorum x E. coli crosses the donor is not contraselected by this medium. Cells capable of utilizing the carbohydrate produce large colonies, differentiating them from both donor and nonutilizing cells which form a veil of background growth.

M-9 Broth (Vaughan, 1962)

2X M-9 Salts--Components were dissolved separately and mixed.

KH_2PO_4	6 g
Na_2HPO_4	12 g
NH_4Cl	2 g
Distilled water	900 ml

2X Casamino Acids

Difco Casamino Acids	30 g
Distilled water	1000 ml
Norite A activated charcoal	2 teaspoons

The solution was allowed to stand overnight at 4C, and was then filtered, and autoclaved.

Preparation of M-9 Broth

2X M-9 salts	500 ml
2X Casamino acids	500 ml
1M $MgSO_4.7H_2O$	2.5 ml
25% NaCl	2.0 ml
40% Dextrose	5.0 ml

Reagents

The production of indole was detected in SIM agar stabs by overlaying the cultures with 0.5 ml chloroform followed immediately by 0.5 ml Kovac's reagent. A deep red color formed in the chloroform layer when indole was present.

Kovac's Reagent

Amyl alcohol	75 ml
Hydrochloric acid (concentrated)	25 ml
p-Dimethylaminobenzaldehyde	5 g

Mutation Procedures

Induction of Mutations with 2-Aminopurine--Approximately 100 cells of a logarithmic culture of the strain to be mutated were inoculated into 10 ml nutrient broth containing 20μg/ml cysteine and 200μg/ml 2-aminopurine. The culture was incubated at 37C with aeration until maximum turbidity developed (Hartman et al., 1962).

Isolation and selection of mutants

Isolation of fermentation mutants--Mutants no longer capable of utilizing specific carbohydrates as carbon sources were detected by plating 10^3-10^4 cells of a 2-aminopurine treated culture on eosin-methylene blue agar plus 0.4% galactose (EMB-gal agar), EMB-mtl agar, and on phenol red agar plus 0.4% xylose (PR-xly agar). Plates were incubated at 37C for 48 hours. When EMB agar was used as a differential medium, the mutant colonies appeared white, dark red, or white with a small dark red center. The mutant phenotype was readily distinguishable from the brilliant green sheen characteristic of fermenting colonies. When phenol red agar was the differential medium, nonfermenting mutants produced red colonies in contrast to the yellow fermenting colonies.

Selection of amino acid and nucleotide mutants by the penicillin method--Mutants deficient in the biosynthesis of amino acids and nucleotides were selected by the penicillin screening method (Davis, 1948; Lederberg and Zinder, 1948; Gorini and Kaufman, 1960). According to this method 0.01 ml of a washed 2-aminopurine treated culture was inoculated into each of two tubes containing 3 ml E medium supplemented with 20μg/ml of cysteine, leucine, and any other amino acids required by the wild type strain. A third tube was inoculated with 0.1 ml of the mutagen-treated strain.

After 60 hours incubation at 37C without aeration 0.1 and 0.5 ml of the contents of each tube were spread onto double enriched E medium. Small colonies, some barely visible, were transferred with sterile toothpicks to nutrient agar plates in a template fashion with 24 colonies per plate. After 48 hours incubation at 37C the nutrient agar master plates were replicated by the velveteen method (Lederberg and Lederberg, 1952) to nine pool agar plates. The plates were incubated at 37C for 48 hours.

The mutant lesion expressed by each colony was indicated by determining the amino acid or nucleotide common to the pools on which the mutant grew. The requirement of each mutant was confirmed by a crystal test as follows. The growth from a pool agar plate was transferred to 3 ml soft agar and poured on to the surface of E agar supplemented with the requirements of the wild type strain. A few crystals of the suspected requirement were placed on the surface of the agar overlay. If the added supplement were indeed required, growth was observed only within the area of the crystals.

In the event that no mutants were found following 2-aminopurine induction, the treated culture was diluted 1:100 in fresh nutrient broth + cysteine + 2-aminopurine, and the entire procedure was repeated.

Often during the course of the study a specific type of mutant - e.g. a tryptophaneless mutant - was required. In such cases an enrichment procedure preceded penicillin selection. A 2-aminopurine-treated culture was washed with saline and diluted 1:10 in E medium to which had been added all the supplements required by the wild type strain plus the amino acid required - e.g. tryptophane - by the type of mutant sought. The enrichment culture was incubated at 37C with aeration until maximum turbidity was reached. This process allowed growth of only wild type and the particular kind of mutant being selected, thereby enriching the proportion of this mutant in the culture.

The enriched culture was washed with saline, and the penicillin selection method described above was followed.

Selection of amino acid and nucleotide mutants by thymineless death--Polyauxotrophic mutants of a thymineless strain resist thymineless death in the absence of the required amino acid because they are not able to initiate new rounds of abortive DNA replication (Maaløe and Hanawalt, 1961). Thymine starvation may thus be used to select doubly auxotrophic mutants (thymine$^-$ amino acid$^-$) in a mutagen-treated thymineless population (Wachsman and Hogg, 1964).

To initiate thymineless death 1.0 ml of a washed, 2-aminopurine-induced suspension of S. pullorum 35 his$^-$ara$^-$xyl$^-$thymine$^-$ was transferred to 9 ml E medium supplemented with cysteine, leucine, histidine, 0.4% glucose, and 1.3% sucrose. The culture was incubated at 37C without aeration. Samples were withdrawn at 12 hour intervals, diluted 10^3, and plated on double enriched E medium supplemented with 20μg thymine, histidine, cysteine, and leucine/ml. After 48 hours incubation at 37C the plates were scored for large (prototrophic) and small (possible auxotrophic) colonies.

Detection of Male Cultures

Altered Surface Properties--Two criteria were used for the detection of the cell surface alterations which characterize donor bacteria: (1) permeability to eosin dye (the staining reaction), and (2) insusceptibility to a female specific S. pullorum bacteriophage.

The Staining reaction--A test of staining reaction consisted of spotting logarithmic cultures (penassay broth) of the bacteria to be tested on eosin-methylene blue agar without fermentable sugar (EMO agar) (Mäkelä et al., 1962). The plates were incubated 6-12 hours at 37C followed by incubation at 25C for a period of 1-7 days. Male cultures

take up eosin dye from the medium and the spot of growth appears pink to red when examined by reflected light. Female cultures remain white.

Each culture tested for a staining reaction was spotted beside a known F^- culture of the same genus, species, and preferrably, also the same strain.

The time of incubation at 37C and at 25C is a critical factor in evaluating the staining reaction and must be standardized for each culture. Several generalizations about the time-temperature relationship can, however, be made. Incubation for 10-15 hours at 37C followed by 24-48 hours at 25C is usually sufficient to distinguish positively from negatively staining E. coli cultures. Rapid growing strains of E. coli - e.g. E. coli 107- on the other hand, require as little as 6 hours incubation at 37C only. S. pullorum cultures require 24 hours incubation at 37C followed by 2-4 days at 25C. Slow growing S. pullorum strains - e.g. S. pullorum 35 chloramphenicol resistant (Cm^r) and 35 $F'lac^+Cm^r$ - require 24 hours incubation at 37C followed by one week at 25C.

In the case of rapidly growing strains, the spots were observed for a color differential every 2 hours. In general, however, plates were examined after 15 and 25 hours at 37C and each day at 25C for seven days.

If no color difference between the control and test spots was observed at the end of one week at 25C, the test cultures were assumed to be F^-. On prolonged incubation at either 37 or 25C both male and female cultures gave a positive staining reaction. Thus, it was always a clear color differential which was sought.

A staining reaction may also be detected in isolated colonies. A positive color, however, requires longer times of incubation at 37 and 25C to develop. A positive staining reaction in a colony was

always confirmed by a spot test of a logarithmic culture grown from that colony.

Insusceptibility to a female specific S. pullorum bacteriophage--Nineteen bacteriophages previously isolated from lysogenic strains of S. pullorum were tested for an ability to distinguish between male and female strains of S. pullorum. Each phage was spotted by loopfuls at its critical test dilution (Adams, 1959) onto S. pullorum 35 F^- and onto 35 $F'lac^+gal^+$, $35F^{+W6}$, and 35 Hfr-1 in tryptone soft agar overlay on tryptone agar plates. After incubation at 37C for 10-12 hours the spots were scored for lysis. Phage 15 was found to be female specific (see results). Phage 15 was spotted on cultures of S. pullorum suspected of being donors in tryptone soft agar. Incubation and scoring were performed as described above.

Transfer of sex factors (Tube mating method)--F^+ and F^- strains were tested for an ability to rapidly donate sex factors to female cultures by the tube mating method. According to this method donor and recipient strains were added to 10 ml penassay broth in a ratio of 1 male to 10 female cells (Jacob and Wollman, 1961) for E. coli - E. coli and for E. coli - S. pullorum crosses to give a final concentration of approximately $2x10^7$ bacteria per ml. The mixture was incubated without aeration for 10-15 hours. A ratio of one donor to one recipient cell was used for S. pullorum - S. pullorum crosses. The mating mixture was then diluted to give 100-1000 colonies per plate and was plated on a medium to contraselect the donor and/or differentiate male and female colonies.

Transfer of chromosomal markers in $F^+ \times F^-$ crosses--A qualitative tube survey method was employed for examining a large number of cultures for an ability to conduct chromosomal markers. A series of 13x100 mm test tubes containing 1 ml penassay broth was inoculated

with various cultures to be tested. The cultures were allowed to attain the logarithmic phase of growth (3-4 hours at 37C). One ml of a logarithmic culture of recipient in penassay broth was added to each tube. The tubes were incubated at 37C for 4 hours to permit genetic transfer and zygote stabilization. After the incubation period loopfuls of the mating mixtures were spotted on media contraselective for the donor and selective for specific recombinants. Plates were incubated for from 48 hours to 4 days, depending upon the selective medium employed. This same method was also used to survey F^+ and F^- strains for an ability to donate and receive fertility factors.

When chromosomal transfer was to be more precisely determined matings were carried out by the flask-shaker method. For matings in which S. pullorum was the donor strain and E. coli the recipient one ml of a logarithmic culture of S. pullorum was transferred to 50 ml penassay broth in a 500 ml Erlenmeyer flask and incubated on a reciprocal shaker at the slowest speed that can be maintained until the logarithmic phase was reached. Five-tenths ml of an exponentially growing culture of E. coli recipient was added to the flask. The mating mixture was incubated at 37C for 12 hours with gentle shaking. The culture was then washed twice with saline and concentrated five fold. One-tenth ml samples were plated on media contraselective for the donor as above.

When strains of S. pullorum were used both as donor and as recipient the inoculation procedure was reversed. One ml of recipient was transferred to 50 ml penassay broth and was grown as above. When the recipient reached the logarithmic phase of growth 5 ml of donor culture was added to the flask. Reversal of the incubation procedure was necessary to obtain the proper ratio of donor to recipient cells.

Curing of F^+ and F' cultures with acridine dyes--Stock solutions of acridine orange and neutral red containing 1 mg/ml distilled water

were prepared immediately before each curing experiment. The tubed dye solutions were wrapped in aluminum foil and autoclaved at 15 pounds pressure for 20 minutes. The acridine dyes were kept in the dark at all times, and all operations prior to the final plating of treated cultures were performed in the absence of white light. Increasing quantities of the stock solutions were added to a series of tubes containing 5 ml nutrient broth adjusted to pH 7.6 and supplemented with 20μg cysteine/ml according to Table V.

One-hundredth ml of a logarithmic culture (penassay broth) of the strain to be cured was transferred to each tube. After incubation in the dark at 37C for 24 hours cells from each tube were streaked for isolated colonies on EMO, EMB-lac, or EMB-gal agar, depending upon the F-factor being removed. EMO plates were incubated at 37C for 24 hours followed by 1-7 days incubation at 25C. EMB-lac and EMB-gal plates were incubated at 37C for 48 hours.

Preparation of Mating Mixtures for Electron Microscopic Examination

Negative Staining with Phosphotungstic Acid (PTA)--A donor and recipient strain were grown separately in penassay broth, centrifuged, and resuspended in M-9 broth. The two cultures were mixed in a ratio of 1:1 v/v and incubated at 37C for 30 minutes. At the end of the incubation period the undiluted mating mixture was mixed 1:1 v/v with 2% PTA. The stained cells were immediately dropped onto formvar covered copper grids with a pasteur pipette. The grids were left undisturbed for 5 minutes. Excess stained culture was removed by touching a piece of filter paper to the edge of the grid. Grids were allowed to dry at 25C.

Positive Staining with Uranyl Acetate (UA)--A 1:1 mixture of a donor and recipient culture prepared as above was dropped onto formvar

TABLE V.--Concentrations of acridine dyes used for curing F^+ and F' cultures.

Concentration of dye in μg/ml	Ml stock solution/ 5 ml medium
0	0.00
2	0.01
4	0.02
8	0.04
10	0.05
12	0.06
16	0.08
20	0.10
32	0.16
40	0.20
60	0.30
80	0.40
100	0.50

covered grids. After standing undisturbed for 5 minutes the grids were floated specimen side down on the surface of distilled water in a watch glass for 5 minutes to remove precipitable nutrients. The grids were then transferred to the surface of 0.5% uranyl acetate in a watch glass for 60 seconds, removed to dry filter paper, and allowed to dry at 25C.

Shadow Casting--Grids prepared as for UA staining were fixed by exposure to the vapors of 2% osmic acid for 2-3 minutes and were then shadow cast with tungsten.

Stained and shadow cast specimens were examined with an electron microscope.

Isolation of High Frequency Recombination (Hfr) Strains

A low frequency recombination culture was grown to the logarithmic phase in penassay broth without aeration, centrifuged, resuspended in an equal volume of saline, and diluted to a concentration of 2×10^8 cells/ml (0.086 O.D. at 420 mμ in a Spectronic 20). The diluted culture was irradiated with an ultraviolet lamp at 20 inches for 20 seconds to give 1% survival. One-tenth ml aliquots of the irradiated culture were spread onto nutrient agar plates. After incubation in the dark at 37C for 48 hours the nutrient agar plates were replicated to selective agar which had been spread with 0.1 ml of a washed exponential culture of recipient concentrated ten-fold. The replicate plates were incubated at 37C for 4 days.

Areas on the donor plates which gave rise to recombinants on selective agar spread with recipient were transferred to penassay broth and incubated at 37C for 12 hours. These possible Hfr cultures were diluted to give approximately 2000 and 200 colonies when plated on nutrient agar plates. The replication process was repeated.

An isolated colony which gave rise to a recombinant colony on selective medium plus recipient was grown to the logarithmic phase in penassay broth. The dilution and replication processes were repeated two more times.

Mating Procedures for Hfr x F^- Crosses

Donor and recipient strains were grown to the exponential phase in penassay broth without aeration. The donor culture was diluted to a concentration of $1x10^8$ cells/ml (0.05 O.D. at 420 mμ). The recipient culture was diluted to $2x10^8$ cells/ml (0.086 O.D. at 420 mμ). The actual number of viable cells per ml of the donor and of the recipient was determined by plating a 10^6 dilution of each culture on nutrient agar plates. Five-tenths ml of the diluted donor culture was added to 4.5 ml of the recipient culture in a 125 ml Erlenmeyer flask. The mating mixture was incubated in a water bath at 37C without disturbance (Hartman et al., 1962).

Gradient of Transmission--The gradient of transmission of genes on the S. pullorum Hfr chromosome was established by plating 10^{-1} to 10^5 dilutions of a mating mixture after 90 minutes uninterrupted incubation at 37C in soft agar overlays on various selective media (Jacob and Wollman, 1961). Plates were scored for the number of recombinants per 100 initial Hfr cells after 72 hours to 7 days incubation at 37C.

Mapping in Time Units (Interrupted Mating)

Syringe method of interruption--One or 1.5 ml samples were withdrawn from a mating mixture at 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120 minutes unless otherwise stated and were transferred to cold sterile serum bottles. Each sample was drawn up and expressed ten times with a cold 2 ml syringe fitted with a 26 guage needle in order to rupture the conjugal bridge and, therefore, the chromosome

(Kitsuji, 1964). The interrupted mating samples were transferred to chilled sterile centrifuge tubes, sedimented at 9750 g for 15 minutes, and resuspended in an equal volume of cold saline. Samples were kept cold at all times to prevent chromosomal transfer (Jacob and Wollman, 1961) and specific pair formation (Clark and Adelberg, 1962).

One-tenth ml aliquots of 10^{-0}, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions were added to 3 ml soft agar and were overlaid onto various selective media. Plates were scored for the number of recombinants per 100 initial Hfr cells after 72 hours to 7 days incubation at 37C.

Waring Blendor Method of Interruption--One-tenth ml samples were withdrawn from a mating mixture at specified time intervals and were transferred to 9.9 ml of cold saline. The diluted mating mixture was poured into a 30 ml capacity Waring blendor cup and blended for 2 minutes at 4C. One-tenth ml aliquots of 10^{-0}, 10^{-1}, 10^{-2} dilutions of the mating mixture were transferred to tubes of soft agar and were overlaid onto selective media as described in the preceding paragraph. Plates were scored for the number of recombinants per 100 Hfr cells after the required incubation period.

Genetic Analysis of Recombinants--Individual recombinant colonies from a given selection were transferred to form the pattern of a grid, regularly arranged on the surface of a plate containing the same medium as that used for their selection. After 48 hours incubation at 37C these plates were replicated to plates containing a variety of selective media which allow the determination of the genetic characteristics of the recombinants. In any selection 100 recombinants were analyzed per time sample. After incubation at 37C for 48 hours the plates were scored for the percentage of the selected recombinants which had received each of the genetic characters analyzed from the Hfr donor (Jacob and Wollman, 1961).

RESULTS

Induction of Mutation with 2-Aminopurine and Selection of Mutants

2-Aminopurine was found to be an excellent mutagen for S. pullorum 35. There was no specificity with regard to the markers mutated. A brief list of the mutants isolated following 2-aminopurine induction is given in Table VI.

Figure 4 presents the data on selection of amino acid and nucleotide mutants by thymineless death. Thirty-six hours incubation of a mutagen treated culture in synthetic medium without thymine permits detection of the largest percentage of doubly auxotrophic mutants.

Survey of S. pullorum Bacteriophages for Male or Female Specificity

Nineteen bacteriophages isolated from lysogenic strains of S. pullorum were surveyed for an ability to distinguish male and female strains of S. pullorum. The results of this survey are presented in Table VII.

Phage 15 at its critical test dilution (2.7×10^5 phage/ml) produced no lysis or very few turbid plaques on male strains but caused confluent or almost confluent lysis of female strains. This phage was used to confirm the maleness of F^+, F', and Hfr cultures of S. pullorum. Phage 15 caused no lysis of F^+ or F^- strains of E. coli W6.

Survey of S. pullorum Strains for Maleness

The most readily detectable criterion of maleness in bacterial strains is a positive staining reaction. Forty-nine strains of S. pullorum

TABLE VI. --Types of mutants isolated following 2-aminopurine induction

Type of mutant of S. pullorum 35	No. of mutants
ara$^-$	24
ara sens.	1
try$^-$	1
thr$^-$	1
35F^{+W6} gal$^-$	1
35F^{+W6} mtl$^-$	1
Hfr - 1 mtl$^-$	1
Hfr - 1 ilva$^-$	1
his$^-$	1
his$^-$ ara$^-$	4
his$^-$ ara$^-$ xyl$^-$	20
his$^-$ ara$^-$ xyl$^-$ pro$^-$	2
his$^-$ ara$^-$ xyl$^-$ thy$^-$	1
his$^-$ ara$^-$ xyl$^-$ mtl$^-$	4
his$^-$ ara$^-$ xyl$^-$ ade$^-$	1
his$^-$ ara$^-$ xyl$^-$ mtl$^-$ thr$^-$	1
his$^-$ ara$^-$ xyl$^-$ mtl$^-$ ade$^-$	1
his$^-$ ara$^-$ xyl$^-$ mtl$^-$ gal$^-$	2
his$^-$ ara$^-$ xyl$^-$ mtl$^-$ gal$^-$ ile$^-$	1
his$^-$ ara$^-$ xyl$^-$ mtl$^-$ gal$^-$ ile$^-$ pro$^-$	1
his$^-$ ara$^-$ xyl$^-$ gal$^-$ mtl$^-$ dex$^-$	1

Abbreviations: ara=arabinose; sens.=sensitive; try=tryptophane; thr= threonine; gal=galactose; mtl=mannitol; ilva=isoleucine + valine; his=histidine; xyl=xylose; pro=proline; thy=thymine; ade=adenine; ile=isoleucine; dex=dextrose.

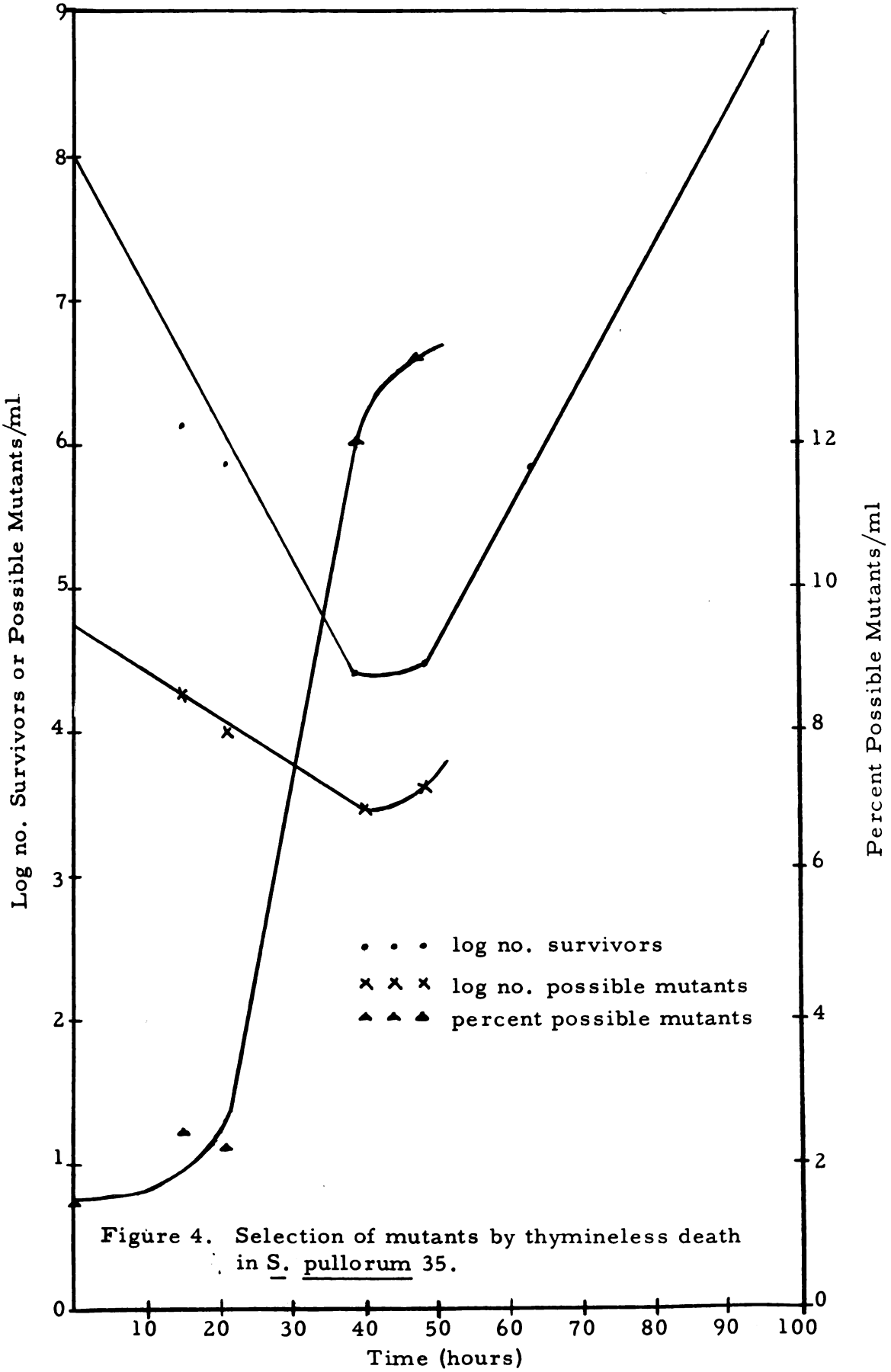

Figure 4. Selection of mutants by thymineless death in S. pullorum 35.

TABLE VII. --Survey of S. pullorum bacteriophages for sex specificity

Strain of S. pullorum	35	35F^{+W6} cured	F'lac^{+}gal^{+} cured	6 cured	35F^{+W6}	Hfr 1	F'lac^{+}gal^{+}	6
Phage								
1	cl	cl	cl	cl	cl	cl	cl	-
4	-	-	-	-	-	-	-	-
5	cl	cl	acl	cl	wkpq	cl	cl	-
6	cl	acl	pq	cl	wkpq	pq	wkpq	-
12	cl	cl	cl	cl	pq	cl	cl	-
15	cl	acl	acl	cl	wkpq	wkpq	wkpq	-
16	cl	cl	cl	cl	wkpq	cl	cl	-
22	cl	cl	cl	cl	pq	cl	cl	-
24	cl	cl	cl	cl	cl	cl	cl	-
26	cl	cl	cl	cl	cl	cl	cl	-
29	cl	cl	cl	cl	pq	cl	cl	-
33	cl	cl	cl	cl	pq	cl	cl	-
34	cl	cl	cl	cl	cl	cl	cl	-
36	cl	cl	cl	cl	cl	cl	cl	-
37	cl	cl	cl	cl	cl	cl	cl	-
38	cl	acl	acl	cl	pq	pq	acl	-
40	cl	cl	cl	cl	cl	cl	cl	-
46	cl	cl	cl	cl	cl	cl	cl	-
50	cl	cl	acl	cl	cl	cl	cl	-

Abbreviations: cl = confluent lysis; acl = almost confluent lysis; pq = plaques; wkpq = very few faint plaques.

were examined for permeability to eosin by spotting them on EMO agar. Forty-eight of these cultures gave a negative staining reaction thus placing them in the category of F^- or F^0. One positively staining strain, S. pullorum 6, was detected. This strain was subsequently shown to contain a sex factor (see below). However, because of its specificity with regard to the nature of recipients with which it will mate S. pullorum 6 was not initially thought to be appropriate to the establishment of a conjugation system in S. pullorum.

Transfer of Sex Factors to S. pullorum

In the apparent absence of a suitable naturally occurring male it was necessary to transfer donor ability to S. pullorum from male strains of E. coli. Two types of sex factors were introduced into S. pullorum 35: (1) the classic F^+ element to permit isolation of Hfr strains with various origins, and (2) an F'lac$^+$ episome for the isolation of an Hfr strain with a predictable order of entry of genetic determinants.

Introduction of an F^+-Sex Factor into S. pullorum 35W--An F^+-sex factor was introduced into S. pullorum 35W from E. coli W6 by conjugating the two strains according to the tube mating method. At the end of the mating period the conjugation mixture was diluted and plated on Levine's EMB agar to give approximately 1000 colonies per plate. Three types of colonies resulted: (1) W6 cells produced large lactose fermenting colonies. (2) S. pullorum 35 cells produced small white nonfermenting colonies and (3) small pink (staining reaction$^+$) nonfermenting colonies. The small pink colonies were transferred to penassay broth, grown to the logarithmic phase, and spotted on EMO agar to confirm their permeability to eosin. Twenty-eight percent of the small pink S. pullorum colonies stained positively in the confirmatory test of staining reaction. These staining reaction$^+$ cultures were

tested for an ability to ferment lactose, an ability to produce indole, and motility. Most of the positively staining cultures possessed the chromosomal genotype of the recipient strain ($lac^-ind^-mot^-$). The S. pullorum 35 staining reaction$^+$ culture which gave the strongest staining reaction was identified as a possible F^+ strain and was designated S. pullorum $35F^{+W6}$, indicating its donor ability and the origin of the sex factor it contains.

The intensity of the staining reaction in S. pullorum $35F^{+W6}$ was initially weak as compared to that observed in F^+ E. coli cultures. As the strain was transferred, however, the intensity of staining gradually increased, suggesting either a host modification of the sex factor and/or a complex nature of the alterations induced by the newly introduced promotor.

The transfer of an F^+ sex factor to S. pullorum 35W is summarized in Figure 5.

Introduction of an F'lac$^+$ Episome into S. pullorum--An F'lac$^+$ episome (the F-promotor with the lactose region of the E. coli chromosome attached) was introduced into a streptomycin resistant mutant of S. pullorum 35 from E. coli AB785 by the tube mating method. The donor was contraselected by plating approximately 1000 cells from the mating mixture on Levine's EMB agar plus streptomycin. Thirty-three percent of the resulting colonies were phenotypically $lac^+mot^-ind^-str^r$staining reaction$^+$.

The stability of the lac$^+$ marker in various S. pullorum 35 str^r lac$^+$ isolates varied. Six percent were unstable yielding 1 lac$^-$:5 lac$^+$ colonies. The remainder segregated 0.05-3.0% lac$^-$ cells. The lac$^-$ segregants stained negatively on EMO agar and could be reinfected with the same F'lac$^+$ particle (see below). Thus, these variants had arisen through loss of the entire F'lac$^+$ particle.

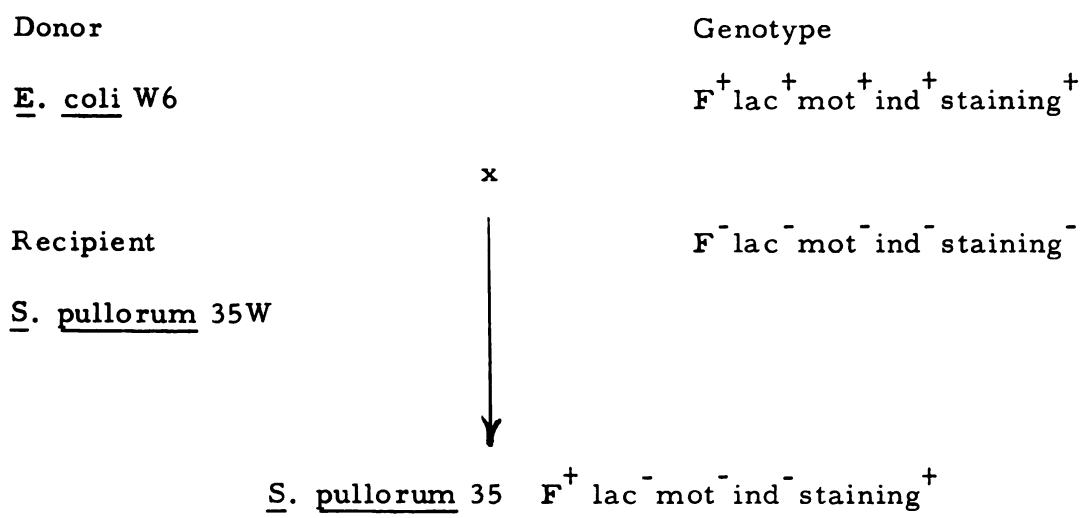

Figure 5. Transfer of an F^+ sex factor to S. pullorum 35W.

The same F'lac$^+$ episome was transferred to S. pullorum 35W according to the tube mating method. Brilliant green agar was used to contraselect the donor and to differentiate lac$^+$ and lac$^-$ exconjugants. The purpose of this transfer was to provide an F'lac$^+$ donor bearing no antibiotic resistant markers thus permiting its ready contraselection in future matings.

Sixty-five percent of the surviving colonies had received an ability to ferment lactose. These fermenting cultures were tested exactly as were the lac$^+$str^r S. pullorum 35 strains and behaved in all cases as did the majority of the str^{r}lac$^+$ cultures with regard to an ability to transfer a sex factor, to be cured, and to conduct chromosomal markers. For this reason only the data concerning S. pullorum 35 str^{r}lac$^+$ cultures are presented in Results.

Evidence of Donor Ability in S. pullorum 35F^{+W6}

Transfer of a sex factor by S. pullorum 35F^{+W6}--A second criterion of maleness in bacterial strains is the ability to transfer fertility factors to F$^-$ cultures, producing positively staining colonies of the parental recipient. S. pullorum 35F^{+W6} was incubated with an E. coli female strain, 104, according to the tube mating method. Approximately 300 cells from the conjugation mixture were spread on each of several EMO agar plates. Thirteen percent of the resulting large pink nonfermenting colonies stained positively, indicating that they had received the genetic determinant of permeability to eosin. The ind$^+$mot$^+$staining reaction$^+$ isolate which gave the strongest staining reaction was designated E. coli 104F^{+35}. The positive staining reaction was readily lost from E. coli 104 F^{+35} during early passages of this culture.

Similarly the positive staining reaction could be transferred to E. coli W6 which had been converted to an F^- strain by treatment with acridine orange (see below) from both S. pullorum $35F^{+W6}$ and E. coli $104F^{+35}$. These $W6F^+$ strains transferred the staining reaction to E. coli 104. S. pullorum $35F^{+W6}$ also transferred the ability to stain to a streptomycin resistant mutant of S. pullorum 35W with an efficiency of transfer of approximately 3%.

$W6F^+$ and 104^+ cultures which received an F-element that had been passed through S. pullorum produced a reduced staining reaction on EMO agar, implying a host modification of the F-element in S. pullorum.

Removal of F^+ Sex Factors with Acridine Dyes--As a third criterion of maleness attempts were made to dissociate the positive staining reaction from the F^+ strain of S. pullorum 35, E. coli W6 (the source of the F^+ element in S. pullorum $35F^{+W6}$), and cultures which had received the F-element from S. pullorum $35F^{+W6}$ by treatment with acridine dyes. The results of treatment of S. pullorum $35F^{+W6}$, S. pullorum 6, E. coli W6, and E. coli $104F^{+35}$ with acridine orange are given in Table VIII.

Acridine orange was effective in removing the F^+ element from E. coli strains but was ineffective in removal of an F^+ element from S. pullorum because of its toxicity for this organism. Two approaches to the problem of curing S. pullorum were investigated: (1) lowering the concentration of the dye below4μg/ml and (2) searching for an acridine dye which would cure but would be less toxic to the S. pullorum strains. The data in Table IX represents treatment of S. pullorum 6 with low concentrations of acridine orange. The staining reaction was dissociated from S. pullorum 6 within a range of 2.6-3.0μg acridine orange/ml. However, because of the narrow range of curing and the

TABLE VIII.--Curing of F^+ strains with acridine orange.

	Concentration of acridine orange in μg/ml												
	0	2	4	8	10	12	16	20	32	40	60	80	100
Strain													
E. coli W6	NC →					5*	5	3	5	0	NG →		
E. coli $104F^{+35}$	NC	8	8	10	10	10	6	2	NG →				
S. pullorum $35F^{+W6}$	NC	NG →											
S. pullorum 6	NC	NG →											

Abbreviations: NC = no curing; NG = no growth
* Numbers indicate the percent cured colonies.

TABLE IX.--Curing of S. pullorum 6 with low concentrations of acridine orange.

	Concentration of acridine orange in μg/ml												
	0	1.0	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0
Strain													
S. pullorum 6	NC →					100*	100	100	NC	NG →			

Abbreviations: NC = no curing; NG = no growth
* Numbers indicate the percent cured colonies.

probability of dilution error when using low levels of dye consistent results were not obtained when the procedure was repeated.

A second acridine dye, neutral red, was tested for efficacy of curing (see Table X). Neutral red proved to be less toxic to S. pullorum strains than acridine orange and was an effective curing agent for both E. coli and S. pullorum. Neutral red in no case increased the percentage curing of a culture; but it did consistently broaden the range of concentrations capable of curing, thus allowing cured colonies to be detected at reasonable levels of acridine dye. All F^+ strains which could be cured with acridine orange could also be cured with neutral red. Figure 6 summarizes the transfer and curing of the staining reaction. All strains which stained positively following conjugation with S. pullorum $35F^{+W6}$ or with staining reaction$^+$ cultures derived from this strain both could be cured of the positive staining reaction and could transfer the permeability to eosin to an F^- recipient.

Low Frequency Transfer of Chromosomal Markers by S. pullorum $35F^{+W6}$--A fourth criterion of maleness of a bacterial strain is an ability to conduct chromosomal markers at a low frequency to a recipient culture. S. pullorum $35F^{+W6}$ was tested for an ability to transfer chromosomal markers to E. coli 104. Following overnight incubation according to the shaker-flask method the washed mating mixture was spread on E-cys agar. This agar permits a natural contraselection of the donor in the presence of a high concentration of E. coli cells, and a selection of $thr^+ leu^+ thi^+$ recombinants. Two hundred-twenty recombinant colonies with the genetic constitution $cys^- thr^+ leu^+ thi^+$ arose. The sex factor contained by S. pullorum $35F^{+W6}$ is thus able to interact with the bacterial chromosome and to conduct chromosomal markers to a recipient strain. This sex factor, therefore, is classified as an episome.

TABLE X.--Curing of F^+ strains with neutral red.

	Concentration of neutral red in μg/ml												
	0	2	4	8	10	12	16	20	32	40	60	80	100
Strain													
E. coli W6	NC →			0.5*	0.5	0.5	0.5	0.5	0.5	3	5	3	NC
S. pullorum 35F + W6	NC	2	4	NC →									NG

Abbreviations: NC = no curing; NG = no growth.
* Numbers indicate the percent cured colonies.

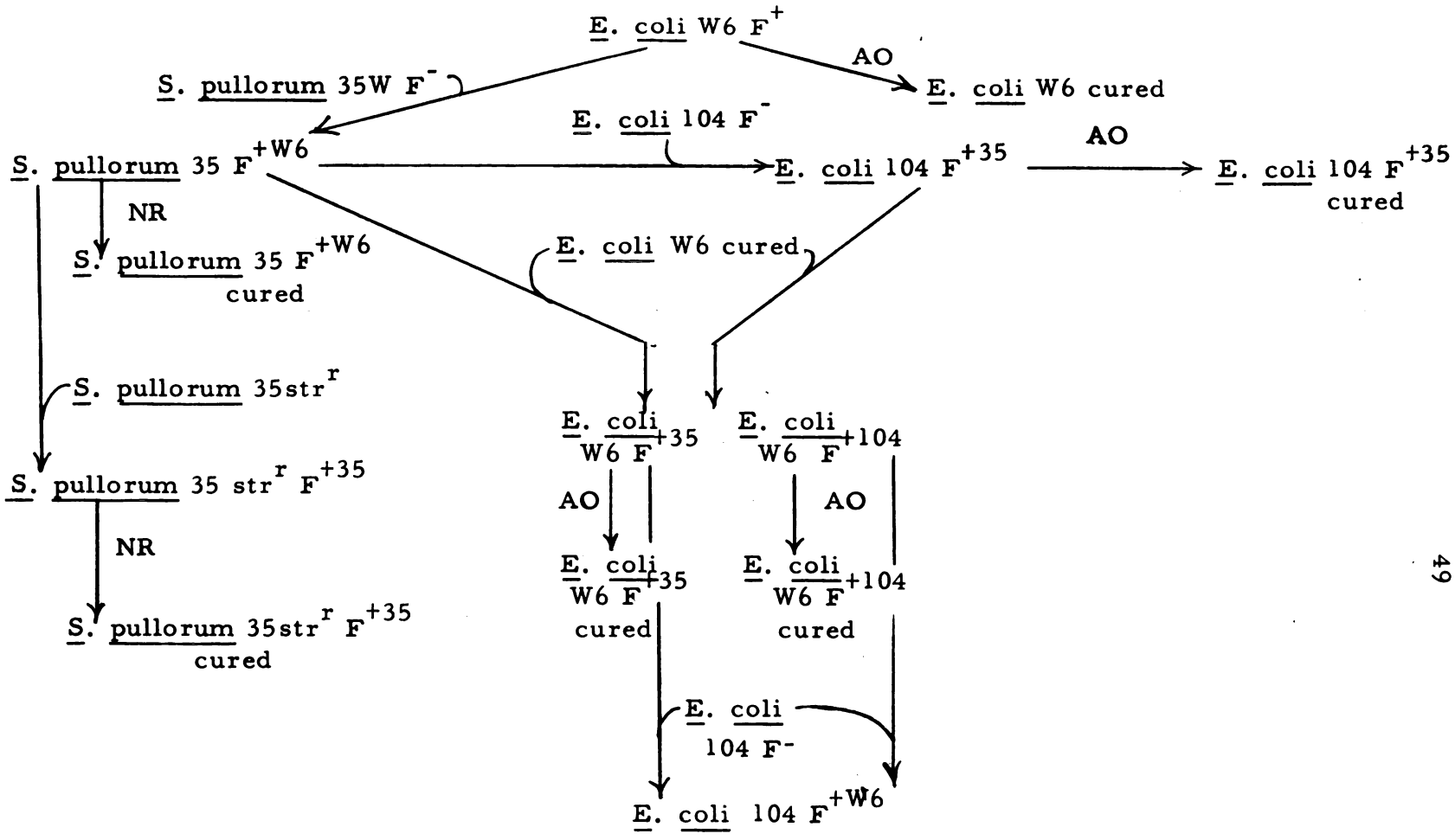

Figure 6. Transfer and curing of the staining reaction.

Abbreviations: AO = acridine orange; NR = neutral red

The frequency of chromosomal marker transfer by S. pullorum $35F^{+W6}$ to E. coli 104 as calculated from Table XI is 2.2×10^{-6} recombinants/donor cell. This figure falls within the range expected of an F^+ culture. The transfer of chromosomal markers by S. pullorum $35F^{+W6}$ to E. coli 104 is summarized in Figure 7.

The ability of S. pullorum $35F^{+W6}$ to conduct chromosomal markers to an E. coli recipient afforded an excellent opportunity to determine the relationship between acquisition and loss of a positive staining reaction and transfer and curing of a sex factor. Table XI presents the results of conjugating positively staining and cured cultures with strains of known mating types. The results presented in this table clarify the relationship between the staining reaction and donor ability of a culture. Those cultures which were assumed to be F^+ on the basis of staining conducted chromosomal markers to F^- strains while the presumably cured strains did not form recombinants with F^- recipients.

Two of the horizontal columns in Table XI require explanation. When E. coli W6 was mated with E. coli $104F^{+35}$ a frequency of recombination five times greater than the value expected in an $F^+ \times F^+$ cross was obtained. The higher frequency of recombinants in this cross is particularly surprising because of the nature of the markers transferred from E. coli $104F^{+35}$. Strain $104F^{+35}$, having received only the sex factor from S. pullorum $35F^{+W6}$, remained genotypically $thr^- leu^- thi^-$. Since a selection was made for $thr^+ leu^+ thi^+$ recombinants, when E. coli $104F^{+35}$ transfers chromosomal markers to F^- mutants in the E. coli W6 population the resulting recombinants are not expressed. The fact that the number of recombinants is less than that observed in the W6 x 104 cross indicates that E. coli $104F^{+35}$ is acting as a donor producing nonviable recombinants. However, the fact that the number of recombinants is significantly greater than expected for

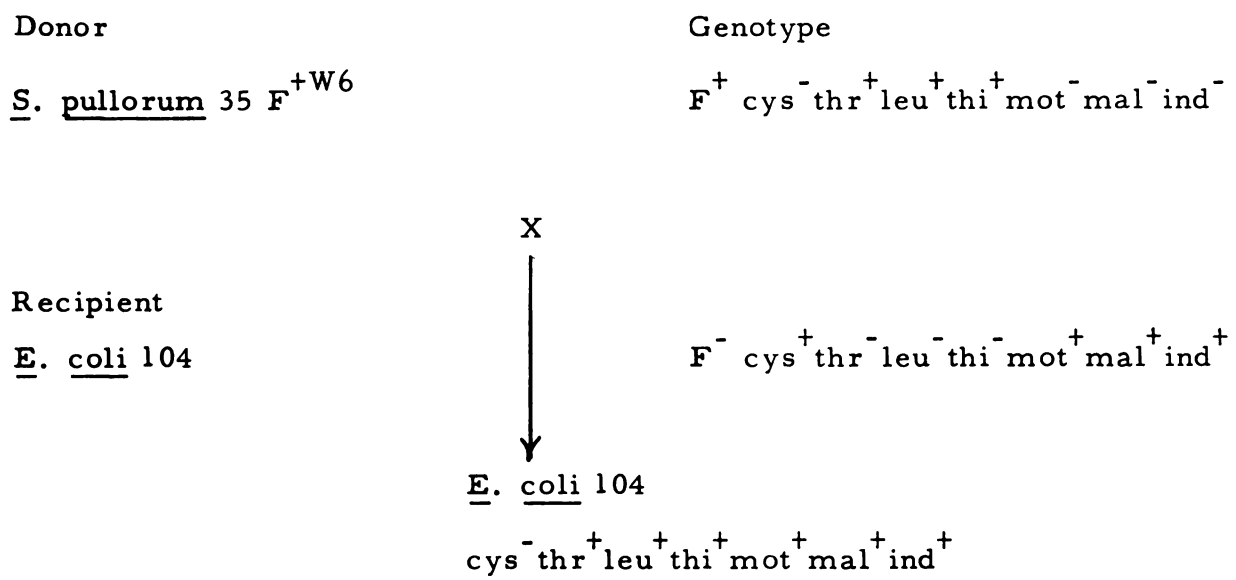

Figure 7. Transfer of chromosomal markers from S. pullorum 35 F^{+W6} to E. coli 104.

TABLE XI.--Transfer of chromosomal markers by positively staining and cured cultures.*

Mating	Mating types	No. recombinants/ml
E. coli x *E. coli*		
W6 x 104	$F^+ \times F^-$	250
W6 cured x 104	$F^- \times F^-$	0
W6 x 104 F^{+35}	$F^+ \times F^+$	120
W6 x 104 F^{+35} cured	$F^+ \times F^-$	200
W6 cured x 104 F^{+35} cured	$F^- \times F^-$	0
W6 F^{+35} x 104	$F^+ \times F^-$	0
W6 F^{+104} x 104	$F^+ \times F^-$	0
S. pullorum x *E. coli*		
35 x 104	$F^- \times F^-$	0
35 F^{+W6} x 104	$F^+ \times F^-$	220
35 F^{+W6} x 104 cured	$F^- \times F^-$	0

*All matings were conducted according to the flask-shaker method. In all cases the donor was contraselected on a medium devoid of an essential amino acid and selective for thr^+ leu^+ thi^+ recombinants.

an $F^+ \times F^+$ cross indicates that the F-factor is readily lost from strain $104F^{+35}$. This, as seen earlier, is the case.

It may also be noted that E. coli W6 cultures made F^+ by receiving a fertility factor which had been established in S. pullorum did not transfer chromosomal markers. Once again a host modification of the sex factor by passage through S. pullorum is indicated.

S. pullorum $35F^{+W6}$ was classified as male strain of the basis of its positive staining reaction, its ability to transfer a sex factor, its ability to be cured with acridine dyes, and its ability to conduct chromosomal markers to an F^- strain at a low frequency.

Evidence of Donor Ability in S. pullorum 6

S. pullorum 6 fulfills all four criteria of maleness: (1) It produces a strongly positive staining reaction, (2) can be dissociated from the staining reaction by treatment with acridine orange (see Table IX), (3) can transfer the ability to stain to E. coli $W6F^-$ or to S. pullorum 35 str^r when conjugated by the tube mating method, (4) and conducts chromosomal markers to a second S. pullorum strain.

There are two points of interest regarding the transfer and curing of a positive staining reaction in S. pullorum 6. First, when S. pullorum 35 str^r was the recipient nearly 50% of the H_2S^+ str^r exconjugant colonies stained positively, indicating an efficiency of transfer 16 times that observed when S. pullorum $35F^{+W6}$ acted as donor. Second, when the E. coli $W6F^-$ made staining reaction$^+$ by mating with S. pullorum 6 was treated with acridine orange 100% curing at concentrations from 16-32μg/ml was affected. Thus, curing occurred at concentrations of dye usually required for an E. coli strain but with a frequency characteristic of S. pullorum 6 (see Tables VIII and IX).

Repeated attempts were made to observe chromosomal transfer from S. pullorum 6 to an E. coli recipient. Three strains, 104, AB 266, and AB 113, and three mating methods--the tube mating, flask-shaker, and gradient of transmission procedures--were used. In no instance were recombinants of any of the possible types selected found. S. pullorum 6 was capable, however, of conducting chromosomal markers during an interstrain conjugation with S. pullorum 35str^{r}his^{-}pro^{-}ile^{-}ara^{-}mtl^{-}gal^{-}xyl^{-} (see below).

Evidence of Donor Ability in S. pullorum 35 str^{r} F'lac^{+} strains

Transfer of an F'lac^{+} particle by S. pullorum 35str^{r} F'lac^{+} cultures--Forty S. pullorum 35str^{r}lac^{+}staining^{+}isolates were tested qualitatively for the ability to transfer the lactose marker to a chloromycetin resistant mutant of S. pullorum 35 by conjugating the strains according to the tube survey method. After the mating period the conjugation mixtures were spotted on PR-lac, Cm(5μgCm/ml) agar. All forty cultures donated the lac^{+} marker at a high frequency, since all spots of the mating mixtures were confluently yellow. Control spots of the recipient strain showed no sign of fermentation. No growth of the donor strain was observed in donor control spots.

The forty S. pullorum 35 Cm^{r}lac^{+} cultures obtained from the previous mating were similarly tested for the ability to transfer the capacity to ferment lactose to S. pullorum 35str^{r}. The matings were carried out as above. The donors were contraselected on PR-lac, str agar. All forty S. pullorum 35Cm^{r}lac^{+} cultures simultaneously donated the lac^{+} determinant and the ability to stain on EMO agar to the recipient strain.

One of the initial S. pullorum 35 str^{r}lac^{+} isolates was analyzed quantitatively for an ability to transfer the lactose marker. This

culture was conjugated with S. pullorum 35Cm^r according to the tube mating method. At the end of the incubation period the mixture was diluted 10^6 and plated on Levines EMB-Cm agar. Lactose positive colonies were indistinguishable from lac$^-$ colonies on this medium. The colonies were transferred to Levine's EMB agar. Thirty-five percent of the Cm^r colonies fermented lactose on Levine's EMB agar.

One of the resulting lac$^+$Cm^r cultures was mated with S. pullorum 35str^r and plated on Levine's EMB-str agar. Four-tenths percent of the resulting str^r colonies were lac$^+$. Two factors appear to be responsible for the low frequency of transfer. The lac$^+$Cm^r cultures consistently grow to a maximum cell count which is one-tenth that of S. pullorum 35str^r; and lac$^+$Cm^r cultures appear to be less efficient in transferring episomic elements to recipient cultures. To compensate for the first of these factors the mating was repeated using a 10-fold concentrated culture of the Cm^r donor. In this second cross 4.6% of the recipient colonies received the lactose character from the donor.

Efficiency of Transfer of an F'lac$^+$ plasmid from S. pullorum 35F'lac$^+$ to S. pullorum 35str^r--S. pullorum 35F'lac$^+$ and S. pullorum 35str^r were mated in a ratio of 1:20 in a 125 ml Erlenmeyer flask. Samples were withdrawn at various time intervals, diluted 10^5, and spread on the surface of Levine's EMB-str agar. After 48 hours incubation at 37C the plates were scored for the number lac$^+$ colonies they contained; and the percent lac$^+$ colonies was computed. The results of this mating are presented in Figure 8. The number of lac$^+$ colonies increased to a maximum value of 85.2% within 25 minutes. This rapid transfer of the F'lac$^+$ plasmid is characteristic of a cytoplasmic factor.

Seventy percent of the recipient population received the F'lac$^+$ factor within 17.5 minutes of the onset of mating. This figure represents approximately three times the interval necessary for 70% transfer of

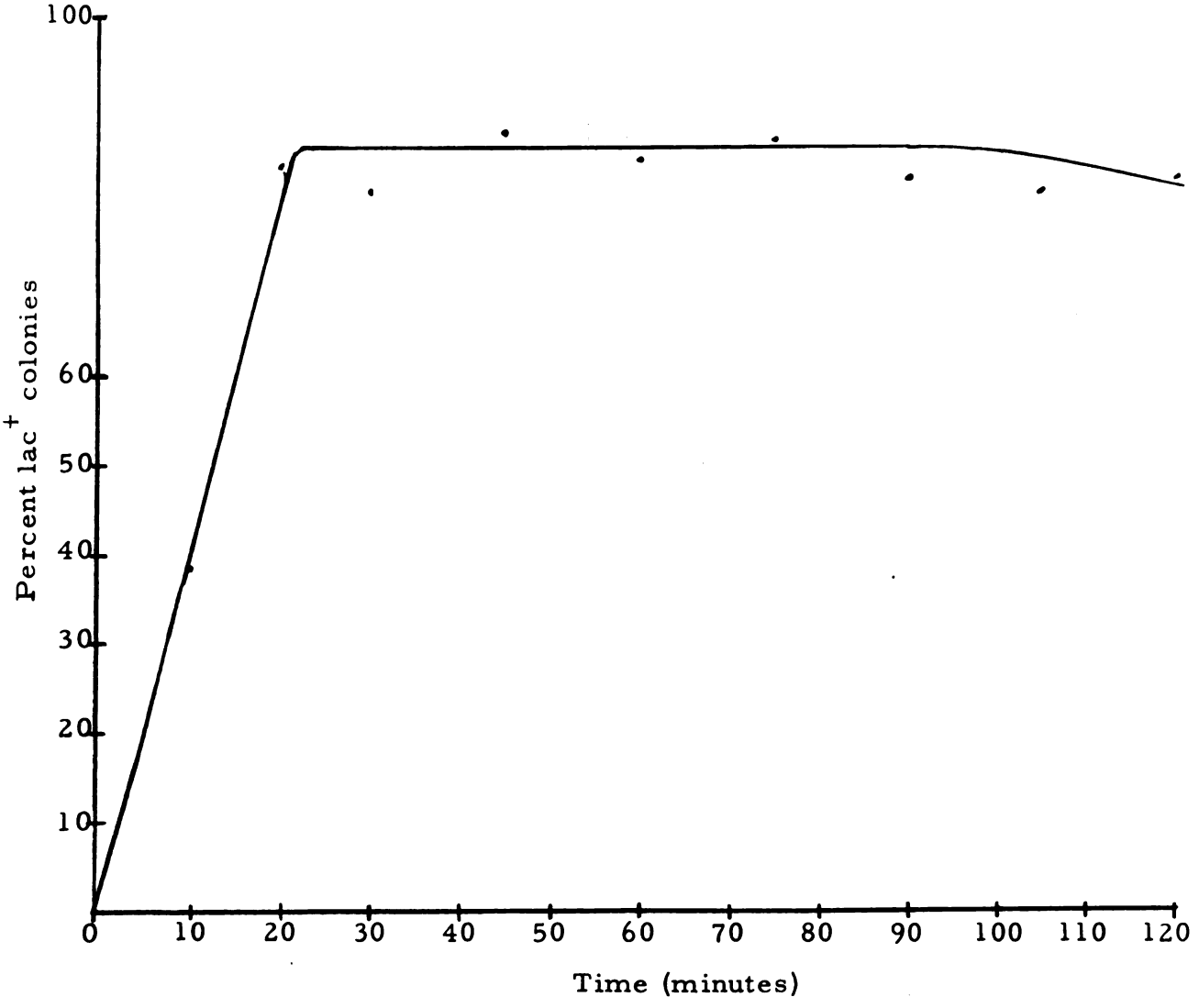

Figure 8. Efficiency of transfer of the F'lac$^+$ plasmid from S. pullorum 35F'lac$^+$ to S. pullorum 35str^r.

an F^+ factor in E. coli and suggests a longer time requirement for the formation of effective pairs in a S. pullorum intrastrain conjugation.

Removal of the $F'lac^+$ factor with acridine dyes--The original donor of the $F'lac^+$ episome, E. coli AB785, spontaneously throws off 1% lac^- segregants. The majority of these segregants retain the staining properties of a male culture. The lac^- segregants are not due, then, to loss of the entire $F'lac^+$ particle. Two possibilities as to the origin of the lac^- segregants remain. The lac^- colonies may arise from a spontaneous mutation of the lactose marker or from a mitotic recombination of the F' episome with the bacterial chromosome carrying a lac^- gene, producing an $F'lac^-$ particle (Jacob and Wollman, 1961). Since the rate of segregation far exceeds the mutation rate of the lactose marker, the second possibility is the more likely.

It is known that the presence of one promotor gene in a cell hinders the introduction of a homologous promotor into the same cell. A lac^- segregant of E. coli AB785 receives an $F'lac^+$ particle whose origin was E. coli AB785 from S. pullorum at a frequency of 0.05% when the two strains are mated according to the tube mating method as compared to a frequency of 33% when an F^- E. coli is substituted for the lac^- segregant.

Three criteria, then, determine whether the loss of an ability to ferment lactose in an $F'lac^+$ strain, no matter if it occurs spontaneously or following acridine treatment, is due to the loss of the $F'lac^+$ or to recombination with the donor chromosome. If the $F'lac^+$ particle is lost the culture will have lost the staining reaction and ability to ferment lactose and may be reinfected with the same F'-element.

The S. pullorum $35str^r lac^+$ and the S. pullorum $35Cm^r lac^+$ cultures which had been used for the quantitative analysis of the transfer

of the lactose character were treated with acridine dyes to determine their ability to be cured. The curing data for these strains are presented in Table XII. Both cultures were simultaneously dissociated from the ability to ferment lactose and to stain on EMO agar by treatment with either acridine orange or neutral red.

Seven cured S. pullorum 35str^{r}lac$^+$ cultures were mated with a tenfold concentrated culture of S. pullorum 35Cm^r by the tube survey method. Similarly fifteen cured S. pullorum 35Cm^{r}lac$^+$ isolates were mated with S. pullorum 35str^r. The mating mixtures were spotted on PR-lac agar contraselective for the donor. All of the cured cultures were reinfected with the same F'lac$^+$ particle they had lost and thus again became lac$^+$ and staining reaction$^+$.

The transfer, curing, and superinfection data are summarized in Figure 9.

Transfer of Chromosomal markers by str^{r}F'lac$^+$ Cultures of S. pullorum--Forty S. pullorum 35str^{r}F'lac$^+$ cultures were tested for an ability to conduct chromosomal markers to E. coli 107. Thirty-nine of the cultures donated only the lactose marker. One exceptional culture donated the ability to ferment both lactose and galactose. Cells receiving these two markers gave a positive staining reaction and were able to transfer both carbohydrate markers and permeability to eosin to a second recipient. On treatment with either acridine orange or neutral red both sugar markers and the positive staining reaction were removed simultaneously. The cured E. coli 107 F'lac$^+$gal$^+$ isolates were reinfected with the F'lac$^+$gal$^+$ particle from S. pullorum 35str^{r}F'lac$^+$gal$^+$ restoring their ability to ferment and to stain. Contraselection of the donor in the reinfection process was accomplished on MB-BCP agar. The spots of mating mixtures on MB-BCP agar were replicated to PR-gal and PR-lac agar to differentiate fermenting from nonfermenting cultures.

TABLE XII.--Curing of F'lac$^+$ S. pullorum strains.

Strain	Concentration of acridine dye in μg/ml													acridine dye
	0	2	4	8	10	12	16	20	32	40	60	80	100	
S. pullorum str^r F'lac$^+$	NC	5*	10	-	-	10	12	7	NG →					AO
	NC	0.07 →		-	2	1	1	3.5	1	NC →				NR
S. pullorum Cm^r F'lac$^+$	NC →						2	NG →						AO
	NC	1	0.05	3	1	4	NC →							NR

Abbreviations: NC = no curing; NG = no growth; AO = acridine orange; NR = neutral red.

*Numbers indicate the percent cured colonies.

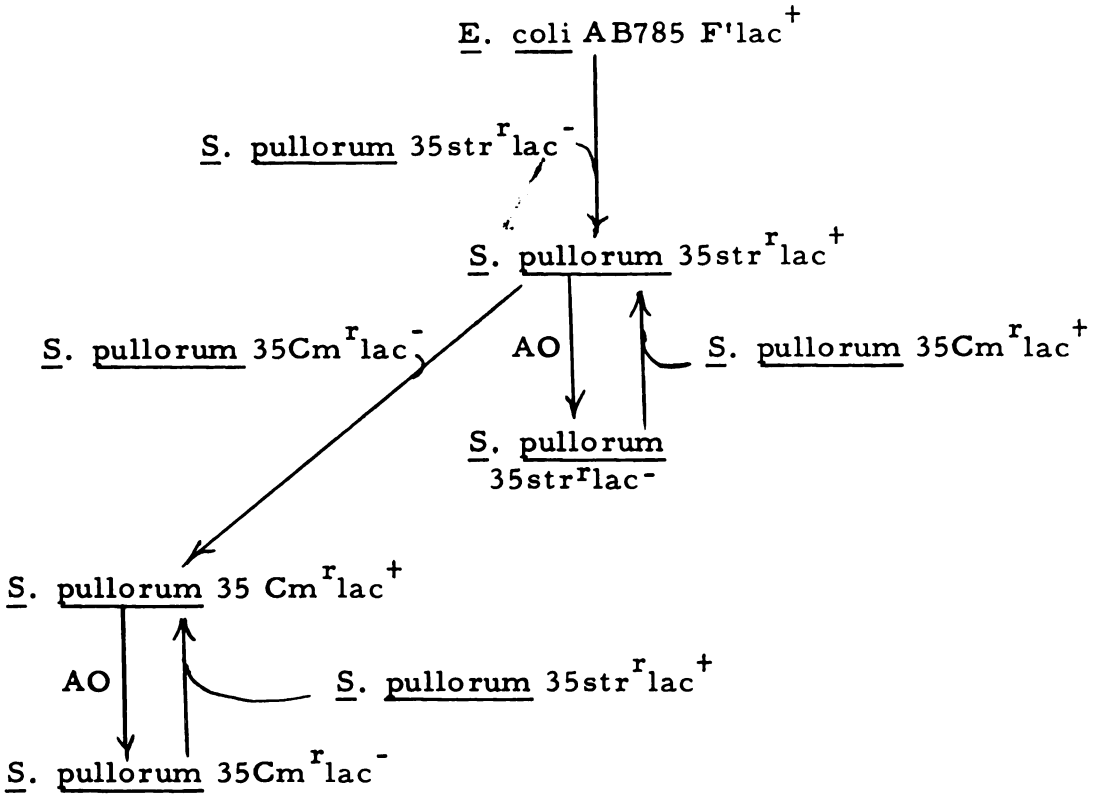

Figure 9. Transfer and curing of F'lac$^+$ particles in S. pullorum 35.

Abbreviation: AO = acridine orange.

It appears, then, that the majority of the lac^+ S. pullorum isolates are $F'lac^+$ and that one isolate carries an $F'lac^+gal^+$ merogenote. Since none of these cultures transfer chromosomal markers, the sex factor they contain behave as plasmids rather than as episomes.

It is not surprising that an $F'lac^+$ particle is not able to integrate into the S. pullorum chromosome. The lac^- marker in the Salmonellae because of its stability is considered to be the result of a deletion. In S. typhimurium all evidence indicates that the entire lactose region of the chromosome is missing (Zinder, 1960b). The mechanism of entrance of an F' particle into the chromosome requires pairing of the chromosomal marker carried on the F'merogenote with homologous region of the recipient chromosome. In the case of a Salmonella carrying a deletion in the lactose region the F' particle is unable to pair with the chromosome and remains cytoplasmic.

It was hoped that the $F'lac^+gal^+$ would be able to integrate into the chromosome, since the addition of the gal marker to the plasmid introduces a region of homologous pairing between the exo- and endogenote. However, repeated attempts to isolate Hfr mutants of the S. pullorum 35 str^r $F'lac^+gal^+$ strain were unsuccessful.

By plating S. pullorum 35 str^r $F'lac^+gal^+$ culture in the stationary phase of growth on Levine's EMB agar it was possible to isolate approximately 0.05% spontaneous variants of the $F'lac^+gal^+$ which were lac^- staining reaction$^+$. These variants on mating with E. coli AB266 according to the tube mating method transferred the staining reaction and the ability to ferment galactose simultaneously. On prolonged incubation of PR-lac agar these lac^- variants fermented lactose. This ability identifies them as $F'lac^-$ cultures of Salmonella very similar to those described by Falkow and Baron (1962) in Salmonella typhosa.

These $F'lac^-gal^+$ variants contain modified sex factors which probably arose from recombination with the donor chromosome.

These sex factors must contain either the lac^- genes of S. pullorum, if they exist, or whatever gene(s) lie adjacent to the gal region in this organism. Thus, the F'lac^+ gal^+ merogenote includes a large region homologous to the S. pullorum 35str^r chromosome. It was anticipated that Hfr mutants might be isolated from this strain.

Evidence of a Conjugation Process

Two criteria determine whether genetic transfer is the result of a conjugation process. First, it must be shown cytologically that cell contact and subsequent steps in the conjugation process do occur; and, second, it must be shown through genetic evidence that cell contact must occur.

Electron Microscopic Evidence of Conjugation between E. coli AB785 and S. pullorum 35str^r--The conjugation process consists of three distinct steps: (1) formation of an effective conjugal pair, (2) conduction of genetic material from the donor to the recipient cell, and (3) formation of a recombinant cell. The first two steps may be evidenced by electron microscopic examination of mating mixtures.

A. Formation of Effective Conjugal Pairs--The formation of effective conjugal pairs proceeds in three stages:

1. Contact between Cells--Figure 10 shows an E. coli and an S. pullorum cell which have established contact. The cell walls of both cells are distinguishable at the point of contact. Contact was also observed to occur between two E. coli cells. Whether these contacts would evolve into effective pairs depends upon the mating types of the two cells in contact. In all likelihood the contact between two E. coli cells would not have resulted in an effective pair, since both cells are of the donor type. However, any donor culture contains a small number of recipient cells, these having undergone a single mutation

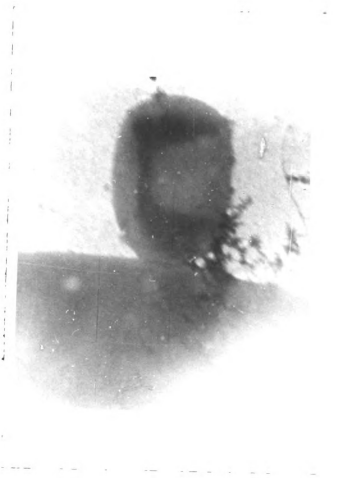

Figure 10. Contact between an S. pullorum and an E. coli cell.

resulting in loss of the sex factor. Thus, two E. coli cells may in some cases form an effective pair.

2. Disappearance of Cell Boundaries--In order to observe the dissolution of cell walls between cells of a conjugal pair mating mixtures were subjected to negative staining by phosphotungstic acid (PTA). There are several advantages to PTA staining. The bacteria are fixed so as to allow air drying at room temperature without producing distortions of the cells or their relationship to one another. PTA fills in those areas of the cell which are empty, making these areas electron dense. Cytoplasmic characteristics and flagella are distinguishable, making them identifying markers.

S. pullorum stained with PTA possesses neither flagella nor fimbrae. The cell wall is thin and difficult to distinguish. E. coli stained with PTA, on the other hand, possesses both flagella and fimbrae, and is approximately twice the size of the S. pullorum cell.

Because of the several structural differences between E. coli and S. pullorum intrastrain conjugal pairs (E. coli-E. coli) may be distinguished from intergeneric (S. pullorum-E. coli) mating pairs.

Both in the case of the homologous E. coli-E. coli conjugation and in the case of the heterologous E. coli-S. pullorum matings (Figure 11) cell walls separating members of the pairs become indistinct in the area of contact.

3. Formation of a Cytoplasmic Bridge--In order to observe the cytoplasmic bridge connecting members of a conjugal pair mating mixtures fixed either by PTA staining or by exposure to the vapors of osmic acid were shadow casted with tungsten. In Figure 12 the bridge between members of an E. coli-S. pullorum pair is distinguishable. The use of a high contrast photographic paper and a long exposure time to emphasize the area of the conjugal bridge has obscured flagellar detail.

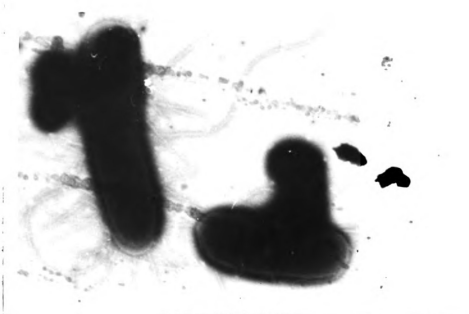

Figure 11. Disappearance of cell boundaries between members of an E. coli-S. pullorum conjugal pair.

Figure 12. Demonstration of a cytoplasmic bridge connecting members of an E. coli-S. pullorum conjugal pair.

Shadow casting was found to be inferior to either PTA or uranyl acetate staining (see below) for demonstration of the conjugation process for the reason that the internal detail of the cells is obscured. After shadow casting conjugation can not definitely be distinguished from cell overlap or cell contact. The ability of PTA staining to detect contact has been indicated above. Figure 13 points out the ease with which cell overlap can be distinguished by UA staining. Uranyl acetate staining proved excellent for demonstrating not only the conjugation bridge, but also the relationship of the donor to the recipient nuclear apparatus.

B. Transfer of Genetic Material--The transfer of genetic material from a donor to a recipient bacterium via conjugation has not previously been observed cytologically (Clark and Adelberg, 1962). An attempt was made to demonstrate nuclear transfer by means of uranyl acetate (UA) staining.

Uranyl acetate is a DNA specific stain. At longer contact times RNA and protein stain lightly (Smith and Melnick, 1962). Uranyl acetate fixes bacteria much as does PTA.

Figure 14 demonstrates the diffuse nature of the DNA in S. pullorum. Figure 13 demonstrates the bipolar arrangement of the nuclear material in E. coli. Since neither fimbrae nor flagella are stained by uranyl acetate, the differences in nuclear aggregation and in size are the only reliable markers for distinguishing the two types of cells.

Figures 15 and 16 demonstrate the transfer of genetic material (UA stained DNA) between members of an E. coli-S. pullorum conjugal pair.

Figure 17 is of some interest in that it raises a pertinent question. Since both cells involved in the pairing are E. coli and they are

Figure 13. Overlapping cells of S. pullorum and E. coli stained with uranyl acetate.

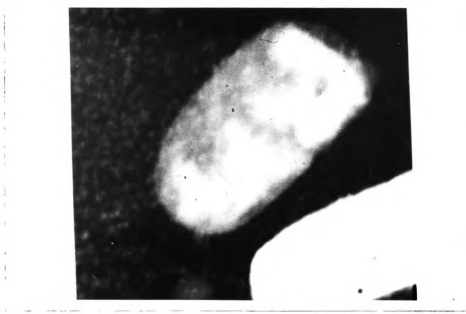

Figure 14. Uranyl acetate stained S. pullorum.

Figure 15. Transfer of DNA between members of an E. coli-S. pullorum conjugal pair.

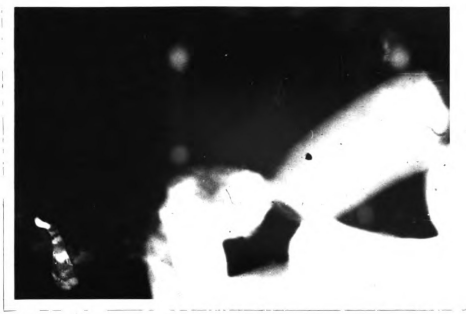

Figure 16. Transfer of DNA between members of an *E. coli*-*S. pullorum* conjugal pair.

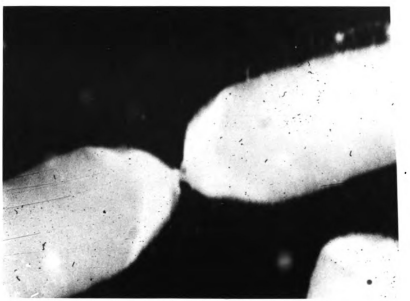

Figure 17. E. coli cells connected by a DNA filled tube.

connected end to end by a DNA filled tube the question arises: does this relationship indicate genetic transfer via conjugation or merely a stage in the division cycle of E. coli? A comparison of Figures 17 and 18 suggests that the association represents conjugation. In this figure the plasmodesma connecting two cells in the terminal stage of division may be seen clearly. The plasmodesma is devoid of UA staining material. Figure 19 shows an E. coli cell at an earlier stage of division. The cell wall separating the two daughter cells had already been laid down at this stage. Thus, during terminal separation one would not expect the DNA of the daughter cells to be continuous but would, rather, expect the plasmodesma to be free of nuclear material.

Figures 20 and 21 show E. coli pairs stained with UA. In all cases a conjugation tube is visible.

Genetic Evidence of Cell Contact

A U-tube experiment was conducted to demonstrate that cell contact must occur between E. coli AB785 and S. pullorum 35str^{r} in order for genetic transfer to occur. The U-tube consisted of the chimneys of two small millipore filters separated by a millipore membrane (420 mμ pore size). Five ml of the donor culture was added to one side of the apparatus and an equal volume of recipient to the other. The fluid from the donor culture was allowed to flow by gravity into the chamber holding the recipient culture. When nearly all of the liquid had drained from the upper section the apparatus was inverted. This process was repeated over a period of 24 hours. At the end of this time the recipient culture was diluted 10^{-6} and plated on Levine's EMB - str agar. No lac^{+} str^{r} colonies developed. A minimum of 200 lac^{+} recipients arose when the experiment was repeated without the millipore membrane separating the donor and recipient cultures and the same dilutions were performed.

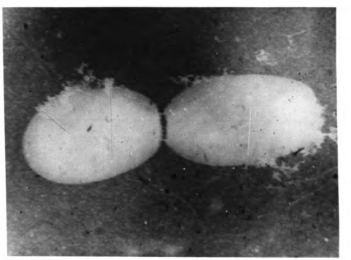

Figure 18. Plasmodesma connecting E. coli cells in a terminal stage of division.

Figure 19. An E. coli cell in an early stage of division.

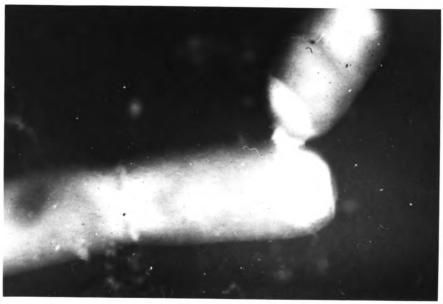

Figure 20. E. coli conjugal pair stained with uranyl acetate.

Figure 21. An E. coli mating pair stained with uranyl acetate.

Isolation of Hfr Strains from F^+ and F' S. pullorum Cultures

A UV-irradiated culture of S. pullorum $35F^{+W6}$ when replicated to E. coli AB266 cells on E-cys agar gave rise to recombinants with the genetic constitution $pro^+ thr^+ leu^+ thi^+ gal^+ mtl^+ cys^- mot^+ ind^+ str^r$ staining$^-$. Areas of the donor giving rise to these recombinants were purified and identified as Hfr variants of the F^+ culture. Four Hfr strains, Hfr-1-4, were isolated. Because the selection was the same in each attempt to isolate Hfr mutants all four gave recombinants of the same genetic constitution and are presumed to have the same origins. Strain Hfr-1 was selected as donor for the genetic analysis of the gross structure of the S. pullorum 35 linkage group. An Hfr variant, Hfr-5, was similarly isolated from an S. pullorum $35F'lac^- gal^+$ culture. Selection in this case was accomplished on E-pro, cys, leu agar.

It is interesting to note that the recombinants formed by mating S. pullorum Hfr-1 with E. coli AB266 contain over half of the genetic constitution of the donor strain. These were shown to be stable recombinants by streaking on EMB-carbohydrate agars. When various Hfr strains of E. coli were used as donor and S. pullorum 35 the recipient stable recombinants carrying a large portion of the donor markers were not formed. The reason for this unidirectional integration of donor determinants remains unexplained.

Recipient Ability of S. pullorum 35

With the isolation of Hfr variants of male S. pullorum 35 strains the S. pullorum conjugation system was supplied with satisfactory donors. It remained to determine whether the entire S. pullorum 35 population would receive genetic material or whether a recipient variant must be isolated.

The recipient ability of S. pullorum 35 was tested as follows. Two matings were performed. E. coli AB785 was conjugated with S. pullorum 35str^r and with S. pullorum 35str^r F' lac$^+$ gal$^+$ which had been cured of the F-factor by treatment with acridine dye. If S. pullorum 35 were an F^0 culture similar to S. typhimurium, then the S. pullorum culture which had received an F'merogenote from E. coli would represent an F$^-$ mutant of the F^0 population, since only an F$^-$ cell may receive genetic material. Thus, one would expect this selected F$^-$, once cured of the sex factor it carried, to receive the F'lac$^+$ factor from E. coli at a higher frequency than would the S. pullorum str^r culture.

The donor and recipient cultures were conjugated according to the tube mating method. Each mating mixture was diluted 10^{-5} and plated on Levine's EMB-str agar. When S. pullorum 35str^r F'lac$^+$ gal$^+$cured was the recipient strain, 1.03% of the resulting colonies were lac$^+$. When S. pullorum 35str^r was the recipient, 1.03% of the surviving colonies received the lac$^+$ determinant from E. coli AB785. S. pullorum 35, then, is an entirely F$^-$ population.

Evidence of Intrastrain Conjugation

The final consideration in the establishment of a conjugation system in S. pullorum was to determine whether a donor S. pullorum strain could conduct chromosomal markers to an F$^-$ S. pullorum culture. The advantage of an intrastrain conjugation system over an intergeneric one is that problems of integration resulting from chromosome nonhomologies are eliminated.

S. pullorum 35F$^+$W6 was mated with S. pullorum 35str^{r}his$^-$ara$^-$xyl$^-$ according to the flask-shaker method. The mating mixture was spread on W-cys,str agar to contraselect the donor. Eighty-eight his$^+$str^r

colonies arose per plate. These recombinants had the genetic constitution $his^+ ara^+ xyl^- str^r$ staining$^-$.

Conjugational Analysis of the S. pullorum Chromosome

Three methods of genetic analysis were employed to determine the order of several genes on the S. pullorum 35Hfr-1 linkage group. These methods, mapping by time units, gradient of transmission, and genetic constitution of recombinants, were used interchangably. The result of each technique was the same--the ordering of the genetic determinants. Choice of a technique was governed by the frequency of recombination and the precise information sought from each mating. Reasons for the selection of a particular method of analysis will become apparent on analysis of the individual experiments.

A recurrent problem during the conjugational analysis of the S. pullorum chromosome was the choice of a medium which would contraselect the donor and select for recombinants of a given type without decreasing the frequency of recombination. In attempting to overcome this problem several selective media and several techniques of selection were employed. Regardless of the selection procedure the order of markers determined was unaltered.

Analysis of the S. pullorum Linkage Group by Intergeneric Conjugation--S. pullorum Hfr-1 was mated with E. coli AB113. At fifteen minute intervals the mating pairs in one ml samples were interrupted by the syringe method. One-tenth ml aliquots of 10^0, 10^1, 10^2 dilutions were plated on E-medium 1 without streptomycin to select for his^+ cells. The donor was not contraselected. After 48 hours incubation at 37C one-hundred his^+ colonies from each time sample were transferred to E-medium 1 without streptomycin and were replicated to a medium selective for str^r cells. Thirty-eight percent of

the his^+ colonies were str^r. The frequency of genetic recombination in this cross was 61.2 his^+str^r recombinants/100 Hfr cells.

The genetic constitution of the his^+str^r colonies was determined by replicating to media selective for $thr^+leu^+thi^+$, gal^+, mtl^+, and xyl^+ recombinants and by transferring each colony to SIM agar. The inheritance of each donor marker among the his^+str^r recombinants was analyzed as a function of time. The results of the analysis of these recombinants are presented in Figure 22. Each marker begins to enter zygotes at a specific time after the onset of mating. The various markers can therefore be arranged in a sequence according to their times of penetration of recipient cells (Hayes, 1964; Jacob and Wollman, 1961).

One feature of Figure 22 requires explanation. Since the leu^- determinant is the first of the three nonselected markers to enter the recipient cell it is expected that this marker should appear most frequently among the his^+str^r recombinants. It appears, however, least frequently. This anomolous frequency results from the nature of the leucine marker in S. pullorum Hfr-1. The leucine marker in S. pullorum Hfr-1 mutates at a high rate from an ability to an inability to synthesize the amino acid. Thus, only a portion of the Hfr population contributes the leu^- determinant to the recipient population.

All of the his^+str^r recombinants tested were $ind^-xyl^-mtl^-$ and gal^-. These markers are not transferred at a high frequency to recipient cells. The absence of these markers in the his^+str^r recombinants defines the proximal segment of the S. pullorum Hfr-1 chromosome as that length of the linkage group carrying genes from the histidine through the cysteine marker. A map of the proximal segment of the S. pullorum chromosome as defined by the four markers tested is shown in Figure 23.

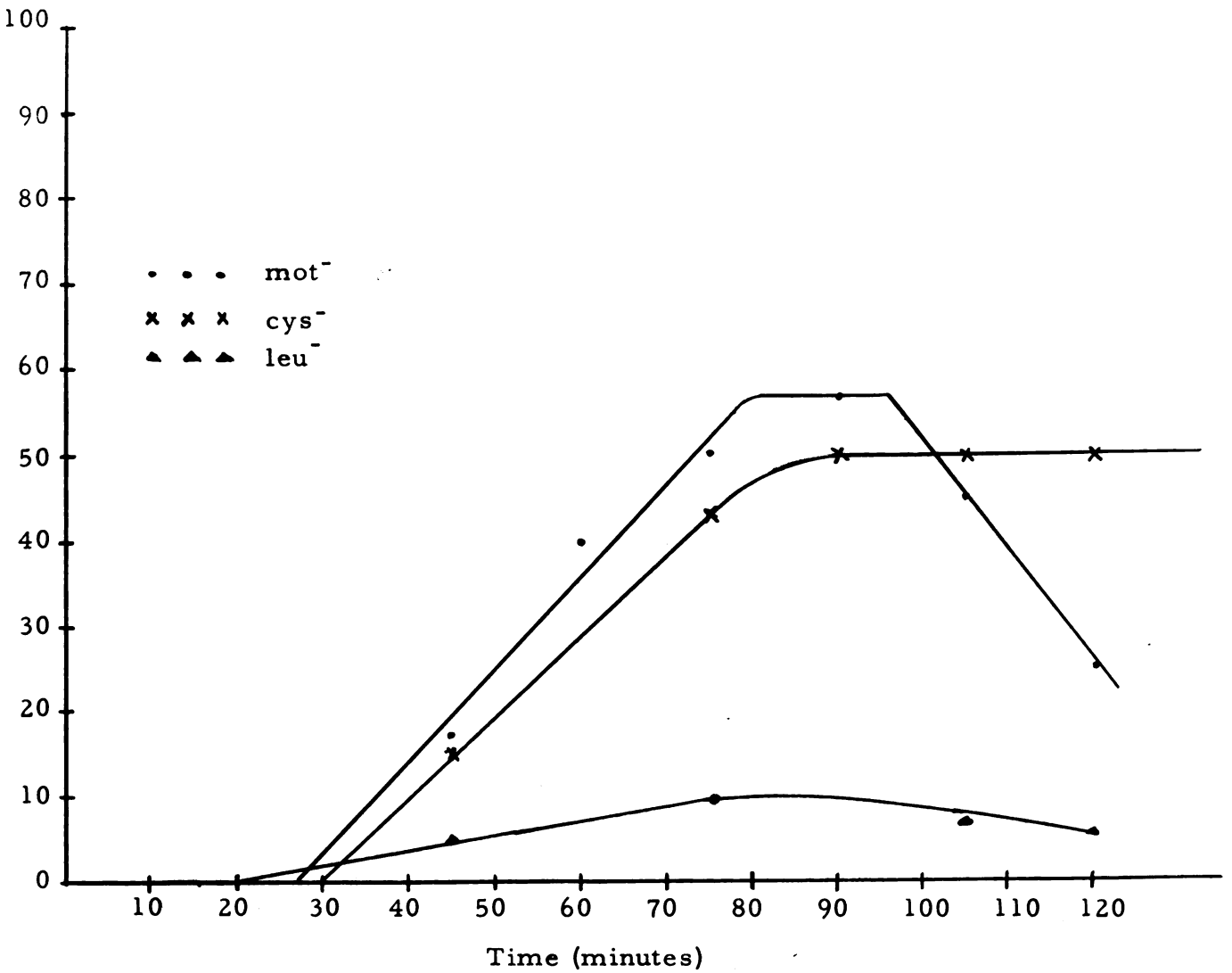

Figure 22. Positions of the mot, cys, and leu genes on the S. pullorum Hfr-1 linkage group as determined by intergeneric conjugation.

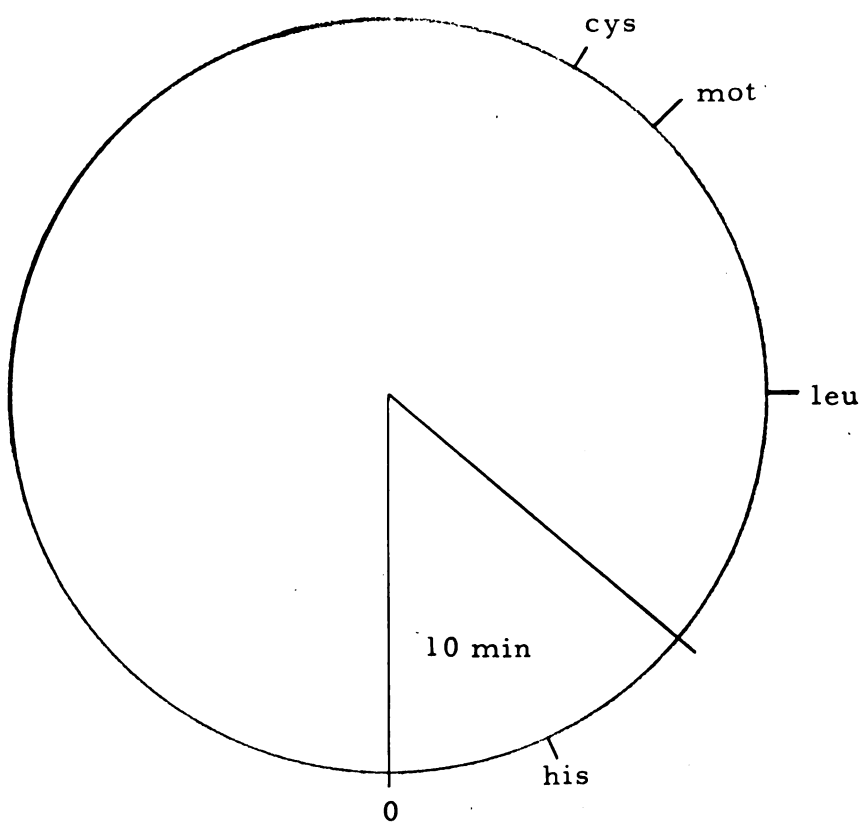

Figure 23. Mapping of the proximal segment of the S. pullorum Hfr-1 linkage group in time units.

The rate of chromosomal transfer from S. pullorum Hfr-1 to E. coli 113 may be determined from the information presented in Figure 22, according to the equation: $\ln p = -kt$, where p = relative frequency of transfer of a marker, k = rate of chromosomal transfer, and t = time of entrance of the marker in minutes (Jacob and Wollman, 1961). The rate of chromosomal transfer in this experiment was 0.022/min.

If the rate of chromosomal transfer is constant during conduction of the proximal segment, then the frequencies or relative frequencies of marker transfer when plotted against time of entrance for each marker should form a straight line (Taylor and Adelberg, 1960). Figure 24 shows the results of such a graphic representation of the data. According to this graph the histidine determinant is expected to penetrate the recipient cell at approximately 5 minutes after the onset of mating.

A second conjugation of S. pullorum Hfr-1 and E. coli AB113 was performed to determine the position of the his^+ marker in time units. In this mating conjugal pairs were disrupted by means of a Waring blendor. The donor was contraselected on W-cys, str agar. Recombinant colonies required 7 days incubation at 37C to develop. Figure 25 shows the results of this cross. Figure 26 describes the alteration of colony forming units of the donor and of the recipient cultures with time in this mating mixture. This information is required to compute the number of recombinants per 100 Hfr cells.

The frequency of recombination in this cross was approximately 0.005 of that observed when selection of his^+ recombinants was performed on E-medium. Thus, W-medium while effective in contraselecting the donor in an S. pullorum-E. coli cross also contraselects a large percentage of the recombinant cells.

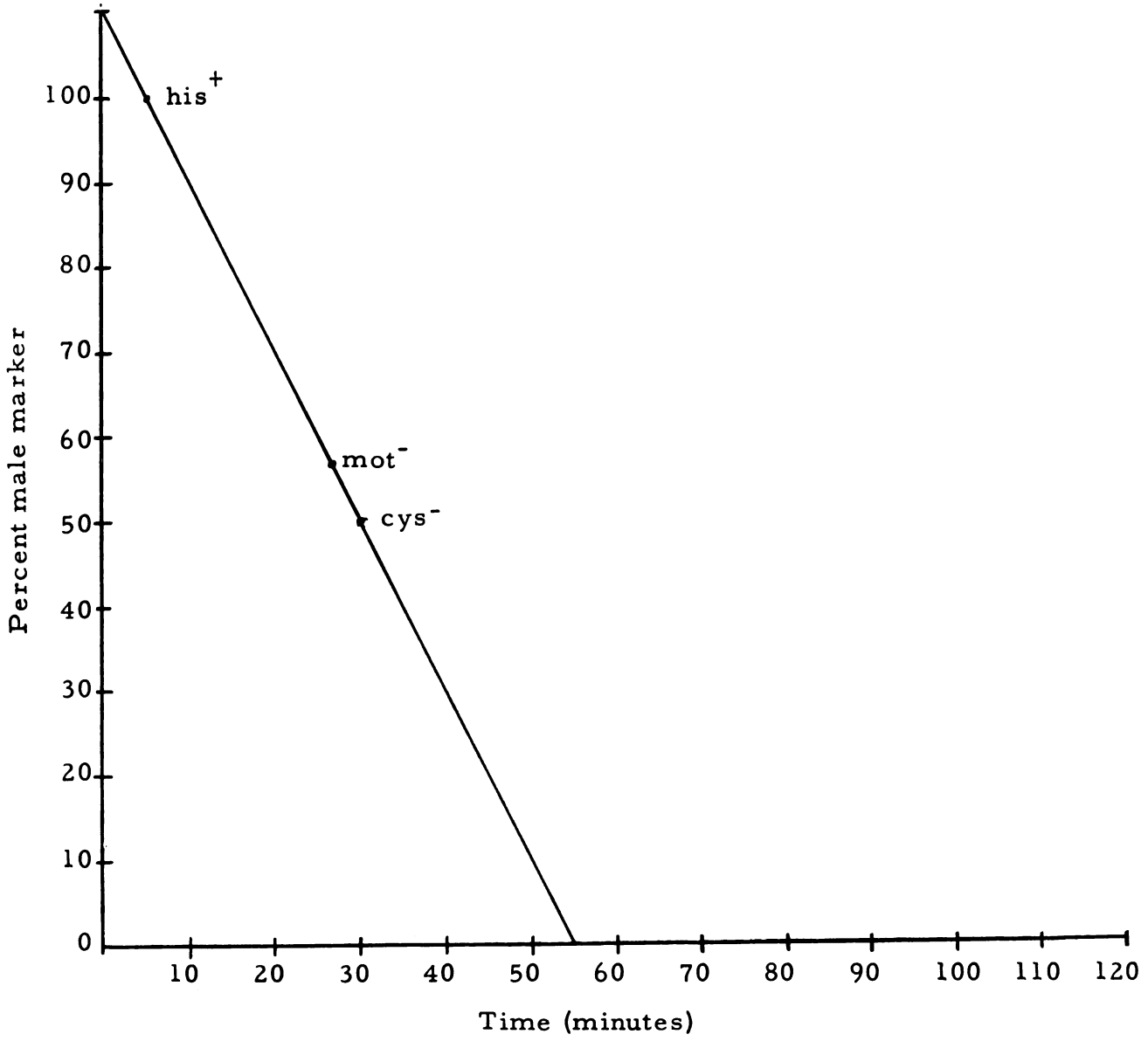

Figure 24. Relationship between percent male marker transfer and time of entry of male markers.

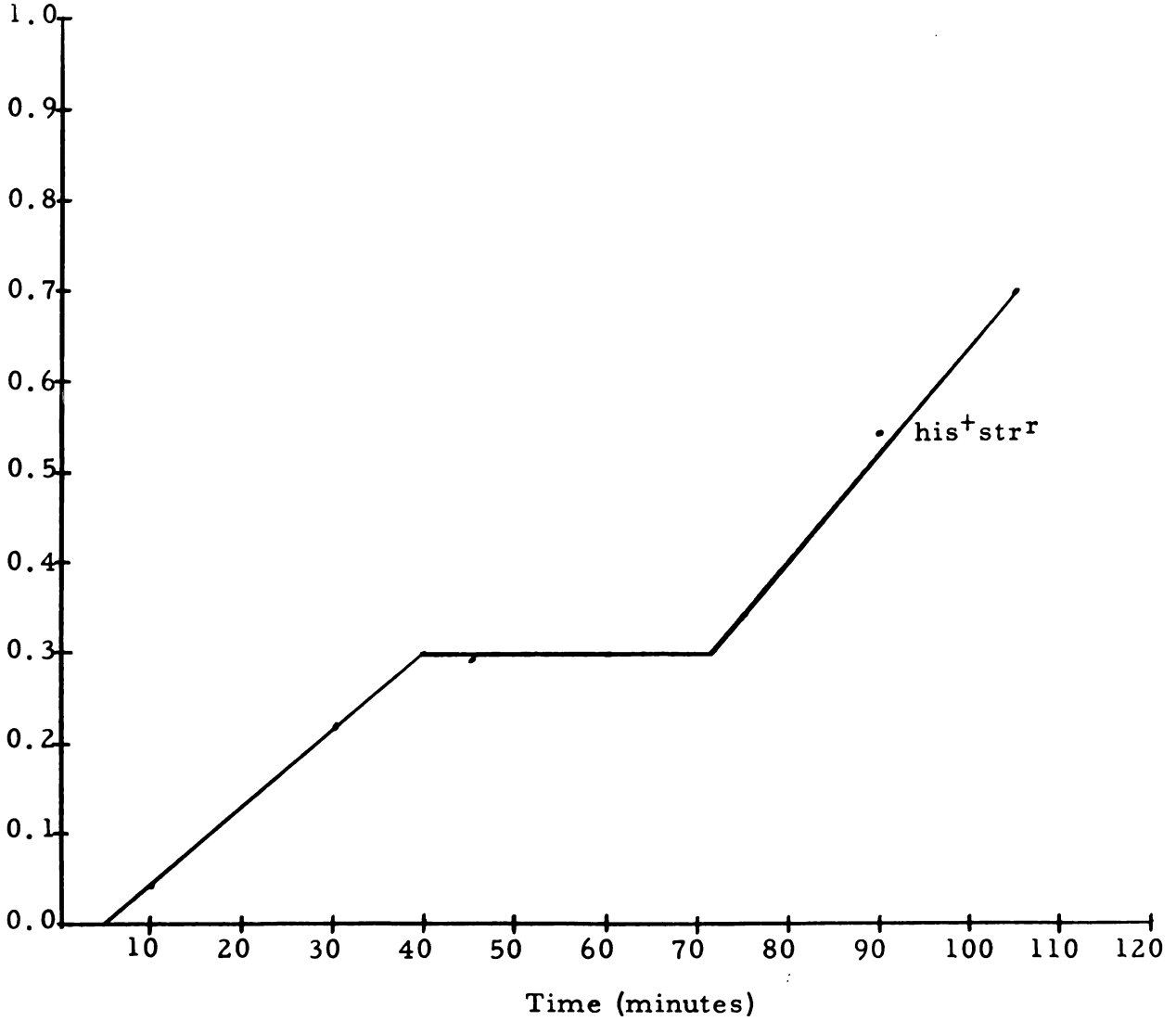

Figure 25. Time of entrance of the his^+ marker into E. coli AB113.

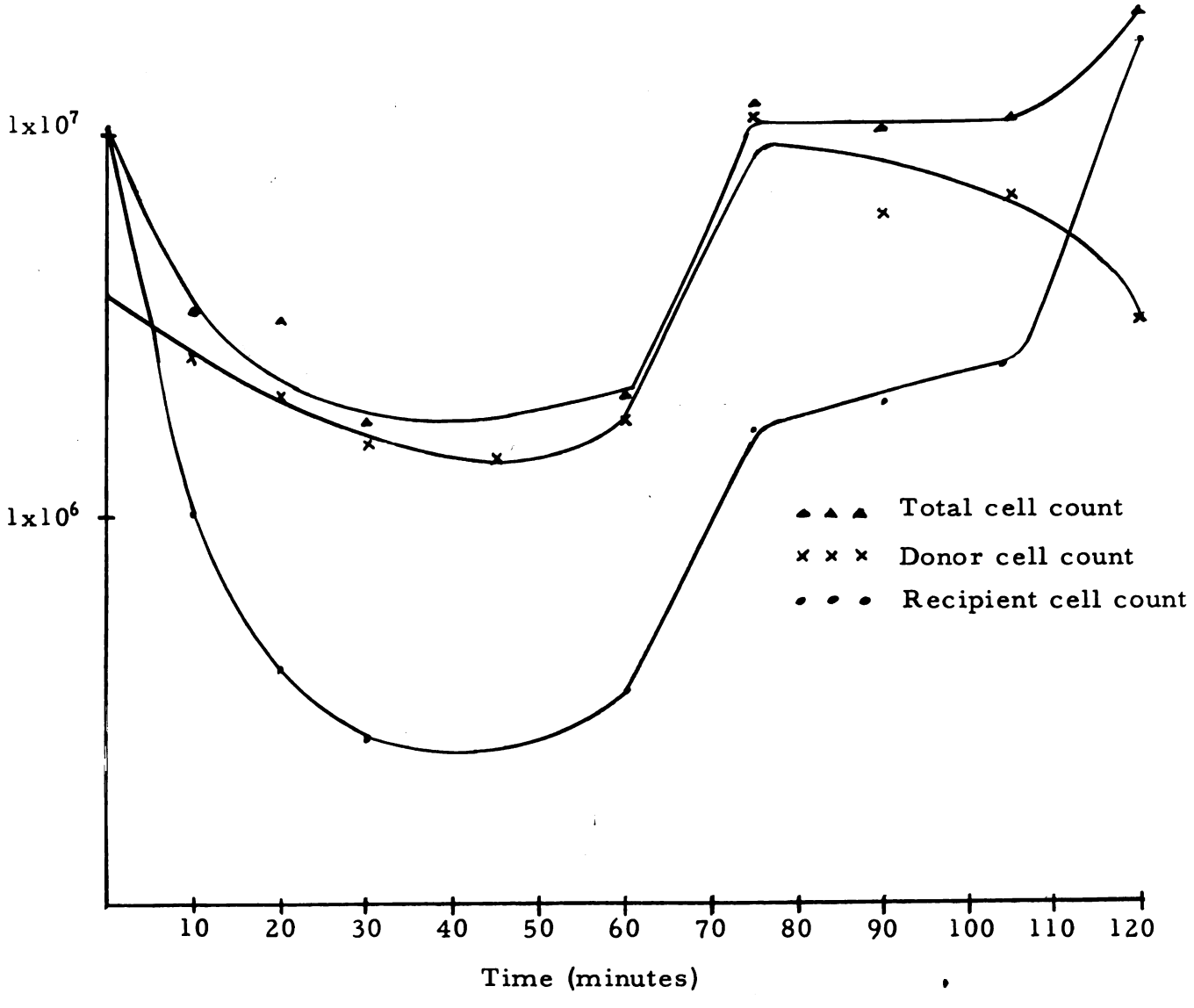

Figure 26. Growth curves of donor and recipient in an S. pullorum Hfr-1 x E. coli AB113 mating mixture.

Analysis of the genetic constitution of the $his^+ str^r$ recombinants at 105 minutes revealed the following percent male markers among the recombinants: mot^- 51.5, cys^- 49.3, leu^- 28.4. The figures for the cys^- and mot^- markers are comparable to those obtained in the previous cross.

Kinetics of Effective Contact between S. pullorum Hfr-1 and E. coli AB113--The kinetics of effective contact were studied by mating S. pullorum Hfr-1 with E. coli AB113, removing samples at specified time intervals, diluting gently 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and plating 0.1 ml aliquotes on media selective for $his^+ str^r$, $gal^+ str^r$, $xyl^+ str^r$, $mtl^+ str^r$, gal^+, xyl^+, mtl^+ recombinants (media 1, 3, 4, 5, 6, 7, 8, respectively). The results of this conjugation are given in Table XIII.

The curves obtained by plotting the number of recombinants as a function of time (Figure 27) indicate the rate of union formation for this particular population density. Each curve starts from the origin, indicating that effective contact formation begins as soon as the cultures are mixed. The curves rise to a plateau value at about 30 minutes. Thus, after 30 minutes uninterrupted contact between the donor and recipient cultures no further specific pairs are formed.

Location of Markers on the Distal Segment of the S. pullorum Hfr-1 Linkage Group--Figure 27 and Table XIII provide information on the location of markers of the distal segment of the Hfr-1 chromosome. The plateau level of recombinants achieved for each selected marker is different. As seen earlier the final frequency with which a male marker penetrates the female cell is inversely proportional to its time of entry. Thus, the various plateau frequencies of transfer represent a gradient of transmission of these male markers to recombinant cells.

TABLE XIII.--Kinetics of union formation between S. pullorum Hfr-1 and E. coli AB113.

Time (min)	No. recombinants/ml						
	$his^+ str^r$	$gal^+ str^r$	$xyl^+ str^r$	$mtl^+ str^r$	gal^+	xyl^+	mtl^+
5	3.8×10^6	5.3×10^4	0	3.6×10^4			
10	7.3×10^6	2.9×10^4	0	1.9×10^5			
15	5.1×10^6	4.8×10^4	0	1.9×10^5			
30	6.3×10^6	7.1×10^4	0	2.6×10^5			
45	2.0×10^7	7.8×10^4	0	1.8×10^5			
60	4.4×10^6	7.6×10^4	0	2.6×10^5			
75	2.8×10^6	1.0×10^5	10	3.2×10^5			
90	2.3×10^6	6.6×10^4	60	2.6×10^5			
105	4.3×10^6	2.6×10^5	60	2.9×10^5			
120	-	4.3×10^5	-	2.3×10^5	2.3×10^7	1.1×10^7	1.5×10^6
150	3.3×10^7	4.9×10^5	40	2.6×10^6	3.1×10^7	2.8×10^7	4.8×10^5
180	4.7×10^7	1.0×10^6	50	2.0×10^6	4.1×10^7	4.5×10^7	1.3×10^6

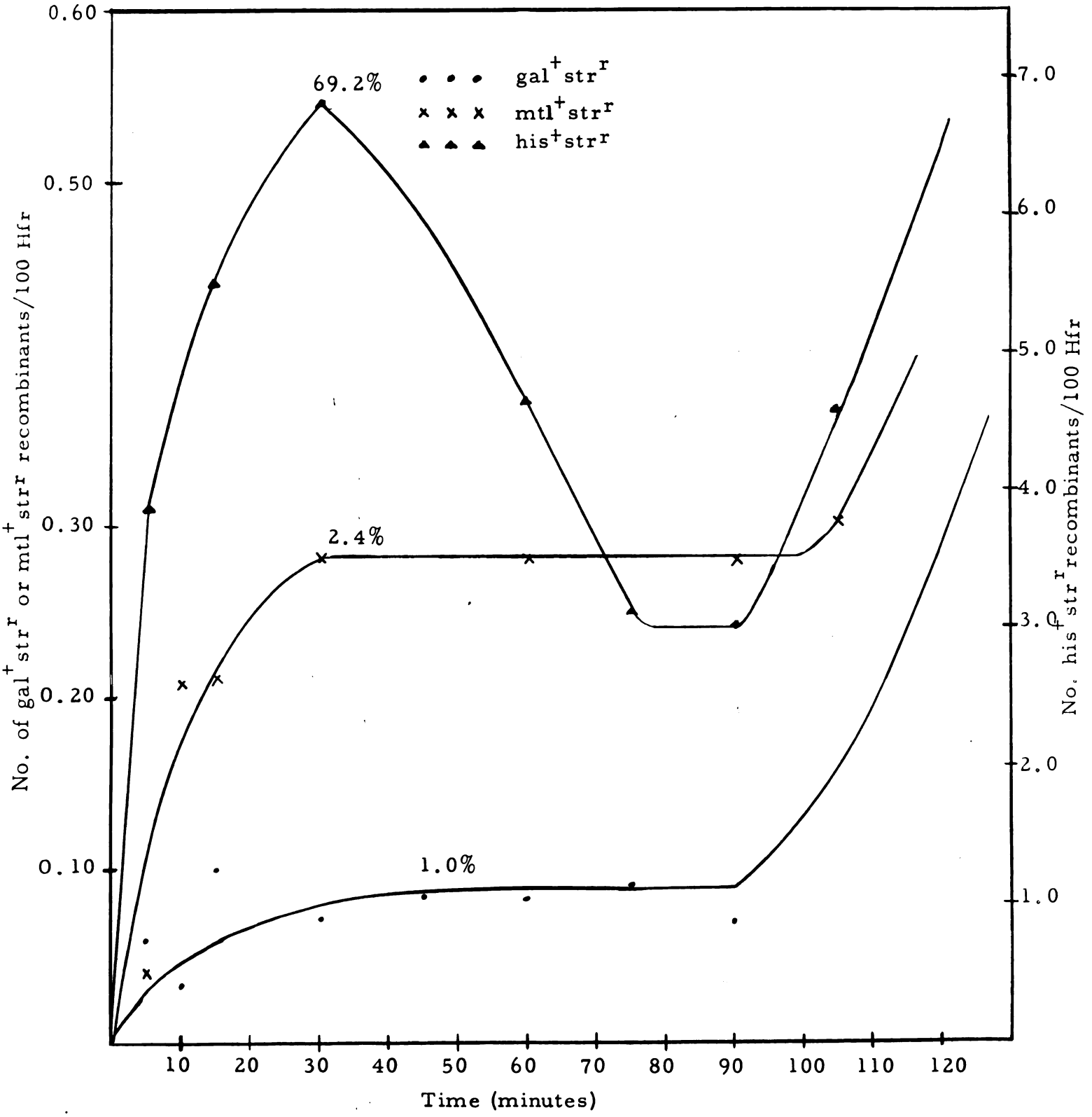

Figure 27. Kinetics of union formation between S. pullorum Hfr-1 and E. coli AB113.

From Figure 27 it may be concluded that the mannitol marker precedes the galactose determinant into the recipient cell and that these two markers lie within a short distance of one another on the linkage group but are at a great distance from the histidine marker.

The low frequency of transfer of the gal, mtl, and xyl markers (see Table XIII) places them on the distal segment of the chromosome. According to Figure 24 these male markers which have not penetrated the $his^+ str^r$ recombinants lie at a distance measured in time units beyond 55 minutes on the donor chromosome.

It should be noted that in Figure 27 after about 90 minutes the number of recombinants of all types in a mating mixture increases sharply and proportionally. This rise is the result of the rapid division of the recombinants in the absence of a selective force.

Table XIII provides one more indication of the order of genes on the distal segment of the S. pullorum Hfr-1 linkage group. By comparing the frequencies of the recombinant classes $gal^+ str^r$: gal^+, $xyl^+ str^r$: xyl^+, and $mtl^+ str^r$: mtl^+ it may be seen that of the three carbohydrate markers entrance of the mannitol determinant is least effected by selection for str^r recombinants. The frequency of entrance of the xyl^+ marker is most effected and that of the gal^+ determinant is intermediate. It appears, then, that the gal and xyl markers are more closely linked to str than is mtl and that xyl is the closer of the two markers to the str locus. It is not possible to determine from these data, however, whether the precise order of these genes on the distal segment is mtl....gal...xyl.str or mtl....gal...str.xyl.

The relative positions of the three carbohydrate markers were confirmed by analysis of the genetic constitution of the $gal^+ str^r$ and the $mtl^+ str^r$ recombinants (Table XIV). According to these data the gal and mtl markers lie close to one another on the linkage group

TABLE XIV.--Genetic constitution of $gal^+ str^r$ and $mtl^+ str^r$ recombinants.

Selected markers	Percent nonselected markers		
	$gal^+ str^r$	$mtl^+ str^r$	$xyl^+ str^r$
$gal^+ str^r$	100	100	39
$mtl^+ str^r$	99	100	0

and the gal determinant lies closer to the xyl region than does the mtl marker.

The order of several genes on the S. pullorum Hfr-1 linkage group as determined by intergeneric conjugation is summarized in Figure 28.

Conjugational Analysis of the S. pullorum Chromosome by Intraspecies Conjugation

An Intrastrain Conjugation with S. pullorum Hfr-1--

S. pullorum Hfr-1 and S. pullorum 35str^{r}his^{-}pro^{-}ile^{-}ara^{-}mtl^{-}gal^{-} were mated according to the gradient of transmission method, diluted 10^{2}, 10^{3}, and 10^{4}, and plated on W-11 and W-12 media. After seven days incubation at 37C the plates were scored for the number of colonies they contained; and the number of recombinants/ml and the frequency of recombination for each character were computed (Table XV). The frequencies of recombination indicate that the entrance of the proline determinant into a recipient cell precedes that of the isoleucine marker. The frequency of recombination for the pro^{+} marker is comparable to that of the his^{+} marker in an S. pullorum Hfr-1 x E. coli AB113 cross. Thus, Hfr-1 behaves as a high frequency recombination donor in an intrastrain as well as in an intergeneric mating.

The genetic constitutions of 100 pro^{+} and 100 ile^{+} recombinants were determined by replicating to E-media or PR-carbohydrate media selective or differential for each of the donor characters. The results of this analysis are given in Table XVI.

Several points become evident on examining Table XVI. First, a true gradient of transmission of donor markers among the recombinants was not obtained, since donor markers on widely separated areas of the chromosome are transferred with the same frequency. The existence of a gradient of transmission of donor characters depends upon the random rupture of the linkage group during its conduction to

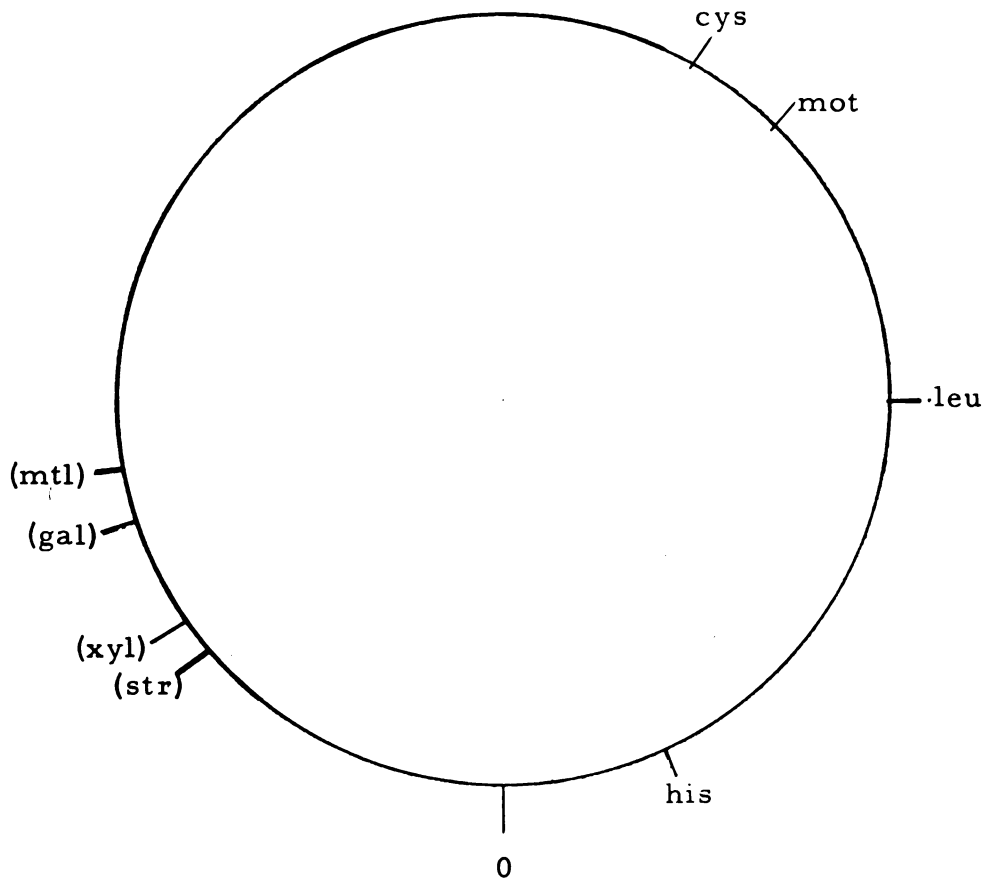

Figure 28. The order of several genes on the S. pullorum Hfr-1 linkage group.

The exact distances between markers in parentheses was not determined.

TABLE XV.--Transmission of markers from S. pullorum Hfr-1 to S. pullorum 35str^{r}his$^-$pro$^-$ile$^-$ara$^-$mtl$^-$gal$^-$.

Selected markers	No. recombinants/ml	No. recombinants/100 Hfr
pro$^+$str^r	1.03×10^4	0.212
ile$^+$str^r	3.3×10^3	0.07

TABLE XVI.--Genetic constitution of recombinants in an Hfr-1 x S. pullorum 35str^{r}his$^-$pro$^-$ile$^-$ara$^-$mtl$^-$gal$^-$ cross.

Selected markers	Percent male marker							
	his$^+$	pro$^+$	ile$^+$	ara$^+$	mtl$^+$	gal$^+$	str^s	staining$^+$
pro$^+$str^r	100	100	100	100	100	100	98	14
ile$^+$str^r	100	100	100	100	100	100	64	16

a recipient cell. In the event that the probability of breakage of the conjugal bridge and chromosome is low, recipient cells continue to receive donor markers until some experimental event such as plating on minimal-streptomycin agar (Jacob and Wollman, 1961) separates the mating pairs.

Stable effective pairs in E. coli have been described by Taylor (1961) as being characteristic of very high frequency (Vhf) male strains. These donor cultures transmit terminal markers with a frequency from 20-200 times higher than the frequency of transmission by ordinary males.

Comparing the relative frequency of transfer of the gal^+ marker from Hfr-1 to E. coli AB113, on the one hand, and to S. pullorum 35 $str^r his^- pro^- ile^- ara^- mtl^- gal^-$, on the other hand, it is evident that the transfer to S. pullorum exceeds that to E. coli by a factor of greater than 100. The same increased frequency of transmission of markers on the distal segment of the linkage group in an S. pullorum intrastrain cross was found for the mannitol and streptomycin markers. Similarly, the pro^+ and ile^+ characters occurred more frequently among the recombinants than was expected on the basis of an Hfr-1 x E. coli AB113 cross. The frequency of recombination for an early marker (e.g. pro^+) was not increased, however, above the value of an early marker (e.g. his^+) in an Hfr-1 x AB113 mating. Thus, it is unlikely that the high values for transfer of late markers is due to an increased ability of the donor and recipient cells to mate but must, rather, be the result of a characteristic of the conjugal pairs once they have formed. It appears, then, that S. pullorum-S. pullorum effective pairs are more resistant to disruption than are S. pullorum-E. coli pairs and that the formation of exceptionally stable mating pairs depends not only upon the characteristics of the donor but also upon the nature of the recipient strain.

A second point of interest evidenced in Table XVI is the occurrence of a positive staining reaction among recombinants formed in an Hfr-1 x S. pullorum 35str^{r}his^{-}pro^{-}ile^{-}ara^{-}mtl^{-}gal^{-} cross. Sixteen percent of the ile^{+} recombinants and 14% of the pro^{+} recombinants gave a positive staining reaction on EMO agar. The ability of these recombinants to stain indicates that they have received promotor genes from the donor strain. All of the staining^{+}pro^{+} recombinants were str^{s}, making them indistinguishable from donor cells. All of the staining^{+}ile^{+} colonies, however, retained their streptomycin resistance. In this second case the staining^{+}ile^{+} recombinants may have received the terminal Hfr gene from the donor and have excluded the str^{s} region of the male linkage group by a recombination event during the formation of the recombinant chromosome. The question of whether the sex factor is always attached to the terminal end of the chromosome or sometimes splits into two parts, one distal and one proximal, both of which are necessary for fertility has not been decided (Jacob and Wollman, 1961). If the second mechanism of integration is possible, the staining^{+}ile^{+}str^{r} recombinants may represent cells which have received only a portion of a donor chromosome containing a split sex factor.

A third fact brought out in Table XVI is a shortcoming of W-media. Since 98% of the pro^{+} and 64% of the ile^{+} recombinants are sensitive to streptomycin, the W-media are not selective for S. pullorum str^{r} cells. The fact that str^{s} recombinants survive on W-media is probably the result of the long incubation time. After 7 days incubation at 37C most, if not all, of the initial streptomycin is inactivated. However, since all of the ile^{+}str^{s} recombinants do not stain on EMO agar, these media do appear to contraselect the donor cells and allow only recombinant colonies to survive.

An Interstrain Conjugation with S. pullorum 6--S. pullorum 6 and S. pullorum 35str^{r}his^{-}ile^{-}ara^{-}mtl^{-}gal^{-}xyl^{-} were mated for 90 minutes. The conjugation mixture was diluted 10^{-2} and 10^{-3} and plated on media selective for ile^{+}str^{r} (W-11) and his^{+}str^{r} (W-10) recombinants. After 7 days incubation at 37C the plates were scored for the number of recombinants they contained; and the number of recombinants/ml and the frequency of recombination for each marker were computed (Table XVII). The recombination frequency for the ile^{+} marker is comparable to that obtained for both an S. pullorum Hfr-1 x E. coli AB113 and an Hfr-1 x S. pullorum 35str^{r}his^{-}pro^{-}ile^{-}ara^{-}mtl^{-}gal^{-} cross. S. pullorum 6, therefore, is classified as an Hfr male. The frequencies of recombination presented in Table XVII indicate that the ile^{+} marker lies closer to the origin of the donor chromosome than does the his^{+} marker.

The genetic constitution of 50 ile^{+} recombinants is given in Table XVIII. Using the same arguments that applied to the Hfr-1 x S. pullorum 35str^{r} recipient cross it may be concluded that the conjugal pairs formed between S. pullorum 6 and a second S. pullorum strain are unusually resistant to disruption. Also, as in the previous mating, all of the staining^{+}ile^{+} recombinants were str^{r} and were also xyl^{-}. The same question as to the position(s) of the staining determinant may be posed in this cross.

One point brought out by this cross but not by the previous intra-strain mating is the relationship between the xyl and str markers. In 96% of the cases whenever a recombinant was str^{r} it was also xyl^{-}; when the recombinants was str^{s} it was also xyl^{+}. The low frequency with which these two donor characters separate indicates that they lie within a short distance of one another on the S. pullorum 6 chromosome. This finding confirms the similar conclusion drawn from an S. pullorum Hfr-1 x E. coli AB113 cross.

TABLE XVII.--Transmission of markers from S. pullorum 6 to S. pullorum 35str^{r}his$^-$ile$^-$ara$^-$mtl$^-$gal$^-$xyl$^-$.

Selected markers	No. recombinants/ml	No. recombinants/100 Hfr
ile$^+$str^r	5.0×10^3	0.43
his$^+$str^r	5.0×10^2	0.043

TABLE XVIII.--Genetic constitution of recombinants in an S. pullorum 6 x S. pullorum 35str^{r}his$^-$ile$^-$ara$^-$mtl$^-$gal$^-$xyl$^-$ cross.

Selected markers	Percent male marker							
	his$^+$	ile$^+$	ara$^+$	mtl$^+$	gal$^+$	xyl$^+$	str^s	staining$^+$
ile$^+$str^r	96	100	100	100	100	78	79	0

Judging from the genetic constitution of the ile^+ recombinants a reasonable order of entry of markers on the S. pullorum 6 chromosome is indicated in Figure 29 below.

O---gal---mtl---ile---ara---his---xyl---str

Figure 29. The probable order of entry of markers on the S. pullorum 6 linkage group.

Abbreviations: O - origin; gal = galactose; mtl = mannitol; ile = isoleucine; ara = arabinose; his = histidine.

DISCUSSION

The establishment of a conjugation system in a species of bacteria which has not previously been shown to be capable of mating depends upon (1) the identification or establishment of a donor culture and (2) the selection of a competent recipient strain. In the event that the conjugation system is to be used for a gross structure analysis of the bacterial chromosome, high frequency recombination (Hfr) mutants of the donor type must be isolated. Each of these Hfr strains should have a different point of origin on the bacterial chromosome so that all markers studied are transferred on the proximal segment of an Hfr chromosome. It is this segment which is conducted to recipient cells at a high frequency and at a constant rate. These characteristics of transfer permit differences in frequencies of recombination for the proximal markers to be readily and accurately translated into time units, representing quantitative expressions of the amount of DNA separating the various genetic determinants.

Early attempts to demonstrate sexuality in the Salmonella were unsuccessful (Zinder and Lederberg, 1952). Later several Salmonella species were found to be receptive to varying degrees to sex-factors of E. coli (Mäkelä et al., 1962). These male Salmonella strains would transfer their newly acquired fertility factors to other Salmonella and, with a greater efficiency, back to E. coli. In all cases the Salmonella strains were male only after mating with a male culture of E. coli under experimental conditions. For this reason the Salmonella have been considered to be universally female strains; and male strains of E. coli K 12 have been referred to as the sole source of maleness in

bacteria (Jacob and Wollman, 1961). More recently an F'lac$^+$ strain of Salmonella typhosa has been isolated from a natural habitat (Falkow and Baron, 1962). This culture contained the same lac$^+$ genes as did an F'lac$^+$ strain of E. coli and is presumed to have derived from that strain by means of an in vivo conjugation.

A survey of forty-nine S. pullorum strains for maleness revealed one male strain, S. pullorum 6. This culture efficiently transferred its maleness and its chromosome to another S. pullorum strain but transferred its sex factor at a low frequency and chromosomal markers not at all to various E. coli recipients. If S. pullorum 6 has received a fertility factor from a derivative of E. coli K12 through an in vivo mating, then the sex factor has become modified so as to be restrictive with regard to the nature of the recipient cultures with which it will effectively conjugate under the experimental conditions used.

The concept of host modification of a sex factor has previously been evoked to explain the stability of an F'lac$^+$ factor in S. abony and the ability of only certain F$^+$ Salmonella to mate effectively with other Salmonella strains (Mäkelä et al., 1962).

In this study the stability of F'lac$^+$ S. pullorum cultures, the intensified staining reaction of S. pullorum 35F^{+W6} and the increased stability of an F$^+$ strain of E. coli 104 following numerous passages, the inability of E. coli W6 F$^+$ strains derived from male cultures of S. pullorum to mate with E. coli recipients may be readily explained as representations of host modifications on F-factors.

Like most Salmonella strains (Mäkelä et al., 1962) S. pullorum 35 readily received F$^+$ and F'lac$^+$ sex factors from E. coli donors. The F$^+$ culture of S. pullorum 35 fulfilled all four criteria of maleness. The F'lac$^+$ strains fulfilled the criteria of a male strain containing a plasmid sex factor.

Several Hfr strains suitable for the analysis of the linkage group have been isolated from F^+ strains of S. typhimurium (Demerec and Sanderson, 1964), and both F^+ and $F'lac^+$ cultures of S. abony have given rise to Hfr strains (Mäkelä, 1962). It was hoped, therefore, that the F^+ S. pullorum 35 culture would yield Hfr strains with various orders of entry and that the $F'lac^+$ S. pullorum culture would permit the isolation of an Hfr mutant with a predictable origin.

An Hfr mutant of S. pullorum $35F^{+W6}$(Hfr-1) with the order of entry O--his--leu--mot---cys--mtl--gal--(xyl)--(str) was isolated. This strain behaved as a classic Hfr in that (1) it transferred a proximal segment of its linkage group with high frequency and a distal segment with low frequency to an E. coli recipient. This Hfr strain was able to form exceptionally stable effective pairs with an S. pullorum 35 recipient and to transfer a large segment of its chromosome at a high frequency during intrastrain conjugation. (2) Hfr-1 conducted donor markers in an ordered and sequential manner and (3) transferred the attached sex factor at a low frequency to recipient cultures.

No Hfr mutants could be derived from any of the many S. pullorum $35F'lac^+$ isolates. These cultures were incapable of conducting chromosomal markers. In this respect they resembled an $F'lac^+$ strain of S. typhosa (Falkow and Baron, 1962) rather than an $F'lac^+$ strain of S. abony (Mäkelä et al., 1962). An Hfr culture was, however, derived from an S. pullorum $35str^r$ $F'lac^-gal^+$ strain. The origin and order of penetration of markers on this donor chromosome was not determined.

S. pullorum 6 was found to be an Hfr strain with the probable order of entry O--gal--mtl--ile--ara--his--xyl--str. This Hfr strain also formed very stable effective pairs with an S. pullorum recipient.

Since S. pullorum 6 transferred the gal^+ marker with a high frequency and the his^+ marker with a lower frequency whereas the S. pullorum Hfr-1 donor conducted the his^+ marker on the proximal

segment and the gal^+ marker on the distal segment of the chromosome, the S. pullorum linkage group must be a closed continuous structure in the F^- and presumably also in the F^+ cell and discontinuous during transfer from an Hfr mutant. Such a circular map conforms with the pictorial representations of both the E. coli (Hayes, 1964; Jacob and Wollman, 1961) and of S. typhimurium (Sanderson and Demerec, 1964) chromosomes.

One of the female strains of S. pullorum, strain 35, was tested for its recipient ability and was found to be a population of entirely receptive cells (F^-) rather than a mainly nonreceptive F^0 culture as is S. typhimurium (Baron et al., 1959). Thus, in its recipient ability S. pullorum 35 closely resembles S. typhosa (Falkow and Baron, 1962) and S. abony (Mäkelä et al., 1962).

Following the isolation of two S. pullorum donors and the identification of a suitable recipient population the order of several genes on the S. pullorum donor chromosome was determined to be his--leu--mot--cys--mtl--gal--str--xyl. The exact positions of the pro and ile markers were not determined; however, it was established that pro lies closer to his than does ile on the Hfr-1 linkage group. The positions of the gal and str markers on the S. pullorum donor chromosome do not coincide with the location of these determinants on the E. coli and S. typhimurium chromosomes (Jacob and Wollman, 1961; Sanderson and Demerec, 1964). A composite picture of the order of genes on the S. pullorum chromosome derived from this study is presented in Figure 30.

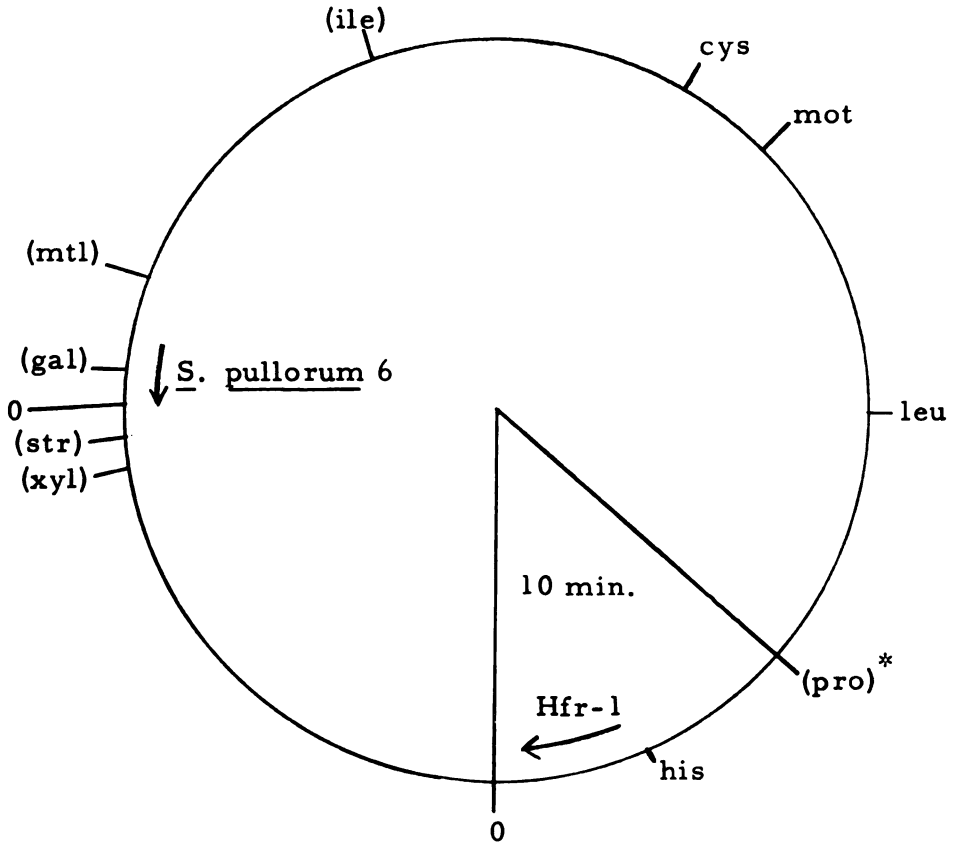

Figure 30. A composite picture of the circular S. pullorum linkage group.

Abbreviations: 0-origin; his=histidine; pro=proline; leu=leucine; mot=motility; cys=cysteine; ile=isoleucine; mtl=mannitol; gal=galactose; xyl=xylose; str=streptomycin.

* The exact position in time units of the markers in parentheses has not been determined.

SUMMARY

Of forty-nine strains of S. pullorum surveyed for maleness one strain, S. pullorum 6, was male. This strain was found to be an Hfr culture and to conduct chromosomal markers to a second S. pullorum strain but not to an E. coli recipient.

One of the female S. pullorum strains was analyzed for its recipient ability both cytologically and genetically. The recipient ability of S. pullorum 35 was genetically characteristic of an F^- rather than an F^0 population. Electromicroscopic studies of E. coli-S. pullorum mating pairs demonstrated the ability of strain 35 to form effective pairs with and to receive genetic material from an E. coli donor.

S. pullorum 35 readily received both an F^+, $F'lac^+$, and an $F'lac^+gal^+$ factor from E. coli male strains. The F^+ and a lac^- segregant of the $F'lac^+gal^+$ S. pullorum 35 cultures fulfilled all four criteria of maleness in bacteria. The $F'lac^+$ isolates exhibited the characteristics of male strains containing a plasmid sex factor.

An Hfr strain isolated from S. pullorum $35F^{+W6}$ conducted chromosomal markers classically to an E. coli as well as to an S. pullorum recipient. Both S. pullorum Hfr-1 and S. pullorum 6 formed exceptionally stable effective pairs with an S. pullorum recipient.

The order of genes on the S. pullorum donor chromosome was found to be his..(pro)..leu,.(ara)..mot..cys..mtl..gal..str..xyl by intergeneric, interstrain, and intrastrain conjugations using S. pullorum Hfr-1 and S. pullorum 6 as donor and either E. coli AB113 or an S. pullorum 35 mutant strain as recipient.

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