GENETIC ANALYSIS OF A "DOUBLE MALE" STRAIN OF ESCHERICHIA COLI K-12

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MATING types of *Escherichia coli* K-12 are defined in terms of ability or inability to effect the transfer of genetic material by conjugation. Ability to effect transfer is conferred upon cells by the presence of a sex factor termed F (LEDERBERG, CAVALLI and LEDERBERG 1952; HAYES 1953). Cells may carry F in either of two states distinguishable by the manner of transfer of F in relation to chromosomal markers. Strains which transfer F independently of the chromosome are known as F^+ strains; strains which transfer F in linkage with the chromosome are known as Hfr strains. In F^+ cells F is carried in an autonomous extrachromosomal state (LEDERBERG 1958), while in Hfr cells F is carried stably attached to the chromosome (JACOB and WOLLMAN 1957).

F^- nondonors may be converted into Hfr donors in a two-step process beginning with the conversion of a given F^- population to the F^+ state by "infection" with F (LEDERBERG *et al.* 1952; HAYES 1953; CAVALLI-SFORZA, LEDERBERG and LEDERBERG 1953). Among the cells of an F^+ population are found spontaneous Hfr mutants, clones of which may be discerned by their ability to transfer chromosomal markers with much higher frequency than clones of F^+ cells (JACOB and WOLLMAN 1956).

Hfr strains transfer chromosomal markers to recipient cells in an ordered temporal sequence beginning with a leading end called the point of origin (O) and ending with the attached sex factor (*sf*) (WOLLMAN, JACOB and HAYES 1956). It has been reported by WOLLMAN and JACOB (1958) that both *sf* and O must be inherited by recombinants of an Hfr by F^- cross in order for the recombinants to express the Hfr phenotype of the donor parent. Any particular Hfr strain transfers markers in a unique temporal sequence, but different Hfr mutants, even those descended from the same F^+ strain, transfer markers in different temporal sequences. For example, one Hfr strain may transfer the sequence O *A B C* . . . . *Z sf* while another, isolated as a mutant of the same F^+ strain, may transfer O *B A Z* . . . *C sf*. In order to explain this observation JACOB and WOLLMAN (1957) proposed that the linkage group of an F^+ strain is a closed curve ("circle") and that an Hfr mutant might be obtained by attachment of the sex factor at any particular point of the curve. Breakage of the linkage group at that point would yield a conventional linear linkage group, terminated at one end by the sex factor. The other end would then become the leading end in transfer.

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Extending this formal genetic picture of *E. coli* K-12, we may ask how we would represent the linkage group of an Hfr carrying two sex factors attached to the chromosome at different sites. Would we still picture only one linkage group in the Hfr or would we need to picture two? By making the common assumption that the linkage group represents a physical structure or chromosome (cf. KELLENBERGER 1960), this question can be restated in a more interesting fashion: if *E. coli* K-12 possesses one circular chromosome and if an attached sex factor breaks this chromosome, two attached sex factors should cause two breaks and the resulting strain should contain two nonhomologous chromosomes.

An Hfr strain possessing two attached sex factors will be termed a "double male." It is the purpose of this paper to describe the preparation and some of the properties of such a strain. A preliminary report of this work has appeared previously (CLARK 1961).

MATERIALS AND METHODS

Media and Mating Conditions: Media for cultivation of cultures and for the selection or scoring of genetic recombinants, as well as the details of the standard conditions for Hfr × F^- crosses, have been fully described by ADELBERG and BURNS (1960). A complex broth consisting of 10 gm tryptone, 5 gm yeast extract, and 10 gm NaCl, dissolved in 1000 ml distilled water and adjusted to pH 7 was used to prepare most liquid cultures. The defined medium used for the selection of recombinants and for particular growth experiments was a half-strength preparation of medium 56 described by MONOD, COHEN-BAZIRE and COHN (1951). Sugars were added to this medium at a final concentration of 0.2 percent. Growth factors were added at predetermined optimal concentrations from 1×10^{-4}M to 2×10^{-3}M. Streptomycin was used at a final concentration of 200 μg per ml.

Cells to be used for mating were grown into exponential phase from an inoculum derived from an overnight broth culture. A dilution of 1:100 in fresh broth supplemented with 0.2 percent glucose was made in an Erlenmeyer flask fitted with a Klett-tube side arm. Densities were measured in the Klett colorimeter and were adjusted to between 1 and 4×10^8 cells per ml. The strains were then mixed in a 125 ml Erlenmeyer flask using 0.5 ml of the minority parent and 4.5 ml of the majority parent. Mating mixtures were incubated at 37°C without agitation generally for 120 minutes. Interrupted mating experiments for determination of transfer kinetics were performed in a 500 ml Erlenmeyer flask with 19 ml of the majority parent and one ml of the minority parent. Interruption of mating with T6 phage was done essentially as described by HAYES (1957). Hfr cells were converted to F^- phenocopies (LEDERBERG *et al.* 1952) by incubating a fully grown broth culture of the strain at 37°C for eight to ten hours with rapid reciprocal shaking (TAYLOR, 1961).

Strains: The strains referred to in this paper are all originally derived from strain K12 of *E. coli* and are listed in Table 1. The following abbreviations are used: *TL*-threonine and leucine dependence (two separate but closely linked

TABLE 1

Bacterial strains

Strain no.	Auxotrophic characters*								Energy-source utilization			Response to phages and streptomycin			Sex	Order of injection (Hfr)
	TL	*pro*	*his*	*ade*	*ser*	*arg*	*met*	*thi*	*lac*	*mal*	*xyl*	*T1*	*T6*	*str*		
JC-158	+	+	+	+	−	+	+	−	+	+	+	S	S	S	♂	O *TL pro lac . . . arg met sf*
JC-12	+	+	+	−	+	+	−	+	−	+	−	R	R	S	♂	O *str mal xyl . . . ser sf*
AB-1116	+	+	+	−	+	+	+	−	−	+	−	?	?	S	♂	See JC-12
AB-1332	+	+	+	−	+	+	−	+	+	+	+	R	S	S	♂	See JC-12
AB-211	−	−	+	+	+	+	−	+	−	+	+	?	R	S	♀	
AB-323	+	+	−	+	+	−	+	−	+	−	−	R	R	R	♀	
AB-325	+	+	−	+	−	−	+	−	+	−	−	R	R	R	♀	
AB-331	−	+	−	+	+	−	+	−	−	−	+	R	R	R	♀	
AB-260	−	+	−	+	+	−	+	−	−	−	−	R	S	R	♀	
D2	+	+	+	−	+	+	+	−	+	+	+	S	S	S	♂	O *TL pro lac . . . ser sf*
															♂	O *str mal xyl . . . met sf*
A14	+	+	−	+	+	+	+	−	?	+	?	?	?	?	♂	See JC-158
A19	+	+	−	+	+	+	+	−	?	−	?	?	?	?	♂	See JC-158
S15	+	+	−	+	+	−	+	−	+	+	?	?	?	?	♂	See JC-12
S16	+	+	−	+	+	+	+	−	−	+	?	?	?	?	♂	See JC-12
S20	+	+	−	+	+	−	+	−	−	−	?	?	?	?	♂	See JC-12
JC-247	−	+	−	+	+	+	+	−	+	−	−	?	?	R	♂	See JC-158

* For abbreviations see text.

loci); *pro*-proline dependence; *his*-histidine dependence;*ser*-serine or glycine dependence; *arg*-arginine dependence; *met*-methionine dependence; *thi*-thiamin dependence; *lac*-lactose fermentation; *mal*-maltose fermentation; *xyl*-xylose fermentation; *str*-response to streptomycin; *T1*-response to phage T1; *T6*-response to phage T6; R-resistance; S-sensitivity; *sf*-sex factor; O-point of origin of genetic material transferred from an Hfr.

Methods: Extracts of DNA were prepared as described by SCHNEIDER (1945), and the DNA determined by the Burton diphenylamine method (BURTON 1956). Protein was determined by the Lowry method (LOWRY, ROSEBROUGH, FARR and RANDALL 1951). Nuclear bodies were made visible by hydrolysis of osmium-fixed cells with 1N HCl and subsequent staining with buffered Giemsa solution (ROBINOW 1942). Total cell counts were made in a Petroff-Hauser counting chamber while viable cell counts (including counts of recombinants) were obtained by pouring an aliquot of the cell suspension, diluted in two ml of soft agar, onto a plate of preset agar. Bacterial dry weights were determined by drying one milliliter of washed cell suspension, containing three to five milligrams of cells, in a small aluminum foil cup and weighing on a Sartorius microbalance.

Symbols: A single colony isolate of any culture is denoted by the original strain number followed by a lower case letter; for example, JC-158a is a single colony isolate of strain JC-158. If the single colony isolate is further purified, this is abbreviated by use of a superscript number following the lower case letter: D2aa = D2a^2 and D2aaa = D2a^3. Since not all experiments were performed with the same isolate of the double male, D2, this notation is introduced to emphasize the relationships of the various isolates.

In order to describe the recombinants of a cross, a notation involving the recombinant markers will be used. In this notation the recombinant markers selected from each parent are separated from one another by brackets with the selected marker transferred from the donor parent outside the brackets; for example, *ser*$^+$(*met*$^+$) recombinants will have received the *ser*$^+$ allele from the donor parent (which was *ser*$^+$ *met*$^-$) and will have inherited the *met*$^+$ allele from the recipient parent (which was *ser*$^-$ *met*$^+$).

Replica plating: Recombinant colonies which were to be scored for unselected markers or fertility were transferred first to agar medium of the same composition as that on which they arose. A large sample of each colony was inoculated onto the "master" plate in the form of a patch, and the master plate incubated until heavy and confluent growth was obtained within each patch. The plate was then replicated onto several test media by the method of LEDERBERG and LEDERBERG (1952). To score for fertility the clones in question were replicated onto a "lawn" of about 2×10^9 F$^-$ cells spread on a medium selective for recombinants. In this case a control plate of the same selective medium without F$^-$ cells was always included in the replication series.

Purification of cultures: New strains, whether obtained by recombination or mutation, were purified by two successive single colony isolations before they were used in further experiments. Normally the colonies were picked from

plates streaked with a dilute cell suspension. In one case purification by micromanipulation of single cells was performed by DR. A. L. TAYLOR according to the method of LEDERBERG (1954). Three such isolates were cultured in broth and from each of these cultures a single colony was isolated. These strains were denoted D2a [4]-1a, 2a, and 3a.

RESULTS

A cross was performed in which one Hfr (JC-12a) was used as donor and another Hfr (JC-158a), as recipient, with selection for recombinants inheriting a marker closely linked to each parental sex factor. Such a cross may be performed unambiguously if one of the Hfr's is converted to an F^- phenocopy (LEDERBERG *et al.* 1952). In this state an Hfr will not effect transfer of genetic material to any potential recipient. Thus in such a cross between two males, one male acts exclusively as a recipient and the other acts as a donor.

The cross performed is illustrated in Figure 1; selection for ser^+ (met^+) recombinants yielded six recombinants for every 10^5 recipient cells mated at a ratio of approximately eight donors for every one recipient. Of 240 such recombinants scored, 60 were ade^- and *T1-S;* these were scrutinized for the predicted properties of a double male. The marker ade^- was needed in the presumptive

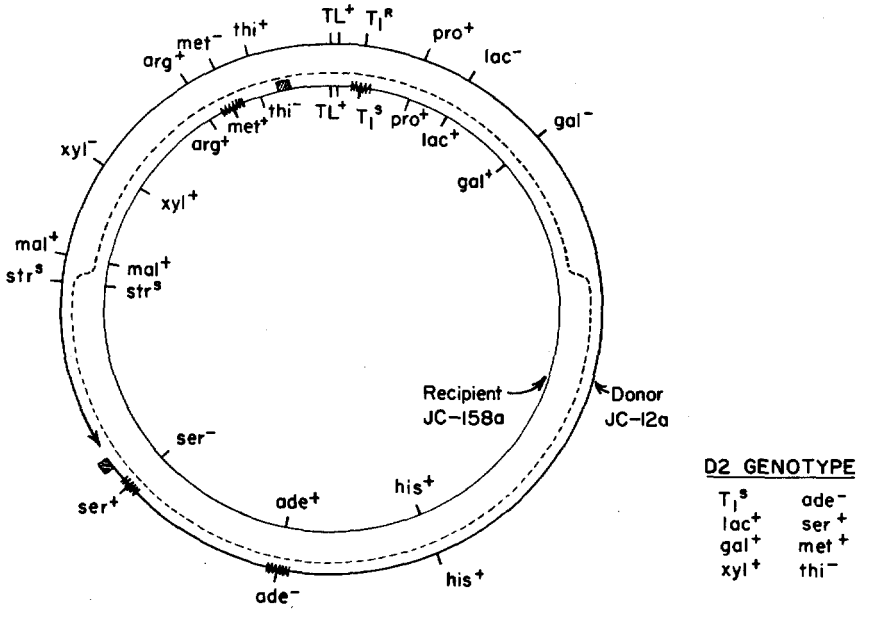

FIGURE 1.—Representation of a hypothetical zygote which would yield a double male from a cross of two Hfr strains, JC-12a and JC-158a. Each solid line represents a parental linkage group, and the dotted line represents a recombinant linkage group leading to a double male of the same genotype as D2. The arrow head represents a point of origin of chromosome transfer and the small block attached to the linkage group represents an attached sex factor. Only recombinants which had inherited the markers designated by a jagged line were checked for inheritance of the sex factors.

double male to provide a nutritional means of selecting against this strain in future crosses. The *T1-S* character was included as a marker linked to the point of origin of Hfr JC-158a so that recombinants inheriting the JC-158a sex factor might express the maximal amount of donor ability associated with this sex factor.

The 60 *ade*$^-$ *T1-S* recombinants were checked for maleness by the method of replicating patches of growth onto lawns of the appropriate F$^-$ spread on selective media. It is theoretically possible to distinguish the two parental male types from the postulated double-male type as is illustrated in Table 2. It was expected that, since *TL*$^+$ and *arg*$^+$ are both markers in the first half of the linkage group injected by JC-12a, they should be transferred with high frequency to zygotes. In crosses with JC-158a, on the other hand, *arg*$^+$ is one of the last markers to enter zygotes and should be transferred with low frequency. Consequently JC-12a should yield a higher recombination frequency for *TL*$^+$ *arg*$^+$ recombinants than should JC-158a and a heavier patch of recombinant growth when replicated onto a *TL*$^-$ *arg*$^-$ F$^-$. In this cross *ade*$^-$ or *ser*$^-$ was to be used as a marker to select against the male parent because omission of these growth factors does not affect the viability of the male. Streptomycin, on the other hand, kills sensitive males as well as any zygote in which the streptomycin-sensitivity allele has been transferred and expressed. Since JC-12a injects *str-S* before *TL*$^+$, it can be expected that the recombination frequency for *TL*$^+$ will be drastically reduced in the presence of streptomycin. This reduction will not occur in crosses of JC-158a because *str-S* is injected long after *TL*$^+$ and only rarely enters zygotes. In a cross yielding *TL*$^+$ (*str-R*) recombinants, therefore, JC-158a should produce a heavier patch of recombinant growth than should JC-12a. The hope was to find a recombinant from the cross of JC-12a by JC-158a which would exhibit high frequencies of recombination under both selective conditions.

In practice it was difficult to distinguish between high and low frequency of recombination by the replica-plating method. Even when control patches of both males were included among the unknowns, the sensitivity of the test had to be increased not only by looking at the amount of recombinant growth resulting from the male inoculum but also by noting the time after replicating when the recombinant growth first was apparent.

All 60 recombinants previously mentioned were males, and many of them appeared to yield heavier recombinant growth under both selective conditions

TABLE 2

Recombination expected from three Hfr strains crossed with AB-260 (F$^-$)

Selected markers		Hfr strain		
from male	from AB-260	JC-159	JC-12	Double male
TL+, *arg*+	*ade*+ or *ser*+	+	++++	++++
TL+	*str-R*	++++	+	++++

Crosses were to be performed by replica plating patches of male growth onto lawns of AB-260 spread onto appropriate selective media. Recombinant growth was then to be scored according to its density within each patch of male inoculum.

than either of the two parents. Twenty-two of these were purified and ten clones of each again tested for maleness. All but two still possessed the desired phenotype. A clone from each was then repurified and five subclones again tested for maleness. When again all were apparently of the desired phenotype, two clones representing two recombinants were crossed in broth with appropriate F^- strains to ascertain the exact amount of recombination for various markers.

One strain (D2) had always shown heavier and faster recombinant growth than any of the others; it and another strain (D16) were, therefore, tested to see if they had inherited the two parental sex factors, i.e., if they possessed a sex factor linked to both *ser* and *arg*. Crosses were made as shown in Table 3. The results obtained are consistent with the hypothesis that D16 has a sex factor linked more closely to *arg* than to *ser* and consequently is a male of the parent JC-158a type, while D2 has a sex factor linked to *ser* as well as one linked to *arg*.

Before a verification of this hypothesis was attempted, strain D2 was purified three times by single colony isolation yielding strain $D2a^3$. Single cells were then isolated by micromanipulation. Three additional strains were thus obtained ($D2a^4$–1a, 2a, and 3a) of which $D2a^4$–1a is representative.

The crosses described in Table 3 were repeated with $D2a^3$, and the recombinant males were isolated. These were crossed in turn to determine for each an order of chromosomal marker transfer; the parent of D2 from which each had inherited its sex factor could then be identified. Some of the data are presented in Table 4. It will be noted that three of the males obtained as *ser*$^+$ recombinants exhibit a recombination frequency gradient similar to the donor parent of D2, JC-12a, whose representative in this series is an independent recombinant AB-1116a. Since the distribution of unselected markers was consistent with the order of injection indicated by the gradient, it can be concluded that these three recombinant males (S15, S16, S20) possess a sex factor linked to *ser* and transmit chromosome with the same point of origin as does JC-12a. In like manner it is seen that two of the recombinant males obtained on selection of *arg*$^+$ resemble parent JC-158a in their recombination frequency gradients. These gradients are also verified by the distribution of unselected markers. It is concluded, therefore,

TABLE 3

Crosses of two presumptive double males to ascertain linkage of sex factor to chromosomal markers

Male	Female	Selection	Recombination frequency	Percent recombinants which were male
D2	AB-325	*ser*+ (*ade*+)	0.52	23
	AB-323	*arg*+ (*ade*+)	1.56	16
D16	AB-325	*ser*+ (*ade*+)	1.29	3
	AB-323	*arg*+ (*ade*+)	0.19	33

Recombinant selection were made from standard 120 minute matings with a male to female ratio of approximately 1:10. In three cases 70 colonies were tested for their inheritance of a sex factor and in one case 115 colonies were tested. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture.

TABLE 4

Crosses which establish the presence of two sex factors in strain D2a³.

Male	Female	Selection*	Recombination frequency	Percent Recombinants male	Strains isolated
D2a³	AB-323	*arg*+(*ade*+)	2.8	28	A-14, A-19
	AB-325	*ser*+(*ade*+)	0.6	30	S15, S16, S20
	AB-331	TL+(ade+)†	2.4	0(≤2.5)	.....

Recombination frequencies for the following selections

Male	Female	*met*⁺(*his*⁺)	*TL*⁺(*his*⁺)	*pro*⁺(*his*⁺)
S15	AB-211	23	9	5
S16		22	13	7
S20		21	8	6
AB-1116‡		21	14	5
JC-158a‡		0.2	30	21
A14		0.2	63	40
A19		0.3	20	13
D2		12	19	14

* Recombinant selections were made from standard 120-minute matings. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture.
† Mating interrupted at 90 minutes with phage.
‡ Adenine contraselections were used for the AB-1116 cross and serine contraselections for the JC-158a cross.

that A14 and A19 have a sex factor linked to *arg* and that they and JC-158a transfer chromosome with the same point of origin.

Data from the cross of D2a³ by AB-331 are recorded in Table 4 to demonstrate that there is no transfer of an autonomous sex factor from D2a³.

This experiment demonstrates that D2 has inherited a sex factor from each of its parents. It remains to be shown that D2 also evinces two points of origin, one corresponding to each sex factor. Two different experiments bear upon this point. In the first, a gradient of recombination frequencies was measured in crosses of the donors D2, D2a³, and D2a⁴–1a by a multiply marked female (AB-331). Selection was made alternatively for several markers, and the markers are ordered from highest to lowest frequency to obtain the order of transfer (JACOB and WOLLMAN 1958). When this is done (Table 5), it is seen that the D2 family

TABLE 5

Comparison of recombination frequencies obtained in crosses of various isolates of the double male with those obtained in crosses of the parents of the double male

Selected marker*	Hfr strain JC-158a	JC-12a	D2	D2a³	D2a⁴-1a
TL+	25	6	23	25	34
his+	3	0.9	1	2	3
mal+	0.2	13	18	12	31
arg+	0.3	10	11	10	22

* Recombinant selections were made from standard 120-minute matings. The recipient strain used in all crosses was AB 331. Serine contraselections were used for the JC-158a cross and adenine contraselections were used for the other four crosses. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture.

are all similar and exhibit what may be viewed as two gradients, one characteristic of each parent; for example, TL^+ recombinants are more frequent than arg^+ recombinants, a characteristic of the gradient obtained with JC-158a, while arg^+ recombinants are more frequent than those which are his^+, a characteristic of the gradient obtained with JC-12a. This then provides *prima facie* evidence that two points of origin are expressed by D2.

The second experiment reveals the times of entry of markers of D2 proximal to the point of origin of the chromosome injected. The results of a cross of D2 by AB-331 are shown in Figure 2; essentially the same results have been obtained using D2a[3]. It is seen that TL^+ enters the zygotes at 14 minutes, while mal^+ follows three minutes later at 17 minutes, and arg^+ even later at 35 minutes. Taking this experiment at face value and interpreting the results as if they had been obtained with a normal Hfr, one can draw a linkage map as follows (WOLLMAN, JACOB and HAYES 1956): $\frac{O \quad TL \quad mal \quad arg}{14 \quad 3 \quad 18}$ . . . This map indicates linkage between *TL* and *mal*, which should be verifiable by noting the number of mal^+ recombinants which are TL^+. Since all mal^+ recombinants should have arisen from zygotes which had also received TL^+ and since *TL* and *mal* are supposedly linked, many mal^+ recombinants should be TL^+: cf. for example the data published for the markers *TL* and *T1*, which are separated with respect to time of entry by three minutes, and show about 70 percent linkage (WOLLMAN *et al.* 1956). The results contradict this expectation (see Table 6), for essentially all mal^+ recombinants are TL^-. The hypothetical map (assuming that D2 is a normal Hfr) also indicates that an increasing fraction of TL^+ recombinants should be mal^+ after mal^+ begins to enter at 17 minutes. This, too, is contradicted by the data reported in Table 6. Thus, it is shown that, although *TL* and *mal* enter zygotes within three minutes of each other, they are not linked. They must, therefore, be entering different zygotes and must repre-

TABLE 6

Analysis of recombinants from kinetic experiment (Figure 2) to show inheritance of unselected markers

Selected marker	Time of sampling (minutes)	Percent* recombinants which were TL^+	Percent recombinants which were mal^+
mal^+	20	0 ($\leq$ 1.2)	. .
	30	2.5	. .
	40	1.2	. .
TL^+	20	. .	0 ($\leq$ 1.2)
	25	. .	0 ($\leq$ 1.2)
	30	. .	0 ($\leq$ 1.2)
arg^+	40	5	50
	45	1	51
	50	5	51

* Eighty colonies of each class were scored for the unselected markers listed.

sent two different points of origin both of which exist in the same population of D2 cells.

It has thus been established that D2 and its descendants possess two sex factors each linked to a different chromosomal marker and two points of origin determined by the chromosomal sites of the sex factors.

One may then ask whether the two sex factors are linked to one another; that is, whether they are both attached to the same linkage group. This question has already been answered by the interrupted mating experiment pictured in Figure 2, for a significant proportion of *arg*$^+$ recombinants are found to be male. The data suggest linkage between *arg* and a sex factor, with a separation of three or four minutes with respect to their time of entry. This sex factor was characterized by isolation of a male from among the 60 minutes *arg*$^+$ recombinants. When mated with a suitable F$^-$, this male recombinant exhibited a JC-158a-type gradient of recombination frequencies (Table 7). The sex factor inherited by this strain cannot, however, have determined the point of origin for the chromosome injection which yielded *arg*$^+$ recombinants at 60 minutes, for, if it had, *arg* would not have begun to enter zygotes until about 100 minutes. Rather, the point of origin must have been determined by the *ser*-linked sex factor originally of JC-12a. Thus at least one male from the D2 population, and probably all those responsible for the *arg*$^+$ recombinants in this experiment, must have had two sex factors attached to the same linkage group.

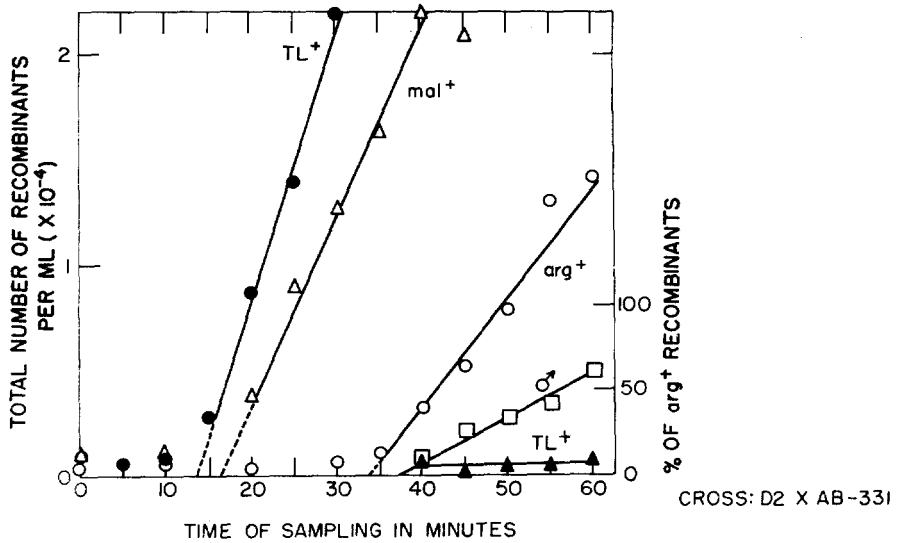

FIGURE 2.—Kinetics of chromosome transfer from double male D2 to recipient AB-331. Mating was performed at 37°C without agitation and was interrupted in samples at five minute intervals with phage. Concentration of males was 1.1×10^7 cells per ml. and the male to female ratio was 1:15. Recombinants were selected for markers *TL*+, *mal*+ or *arg*+. The *arg*+ recombinants were scored for maleness and *TL*+ as unselected markers. The distance between *mal* and *arg* approximates the distance between these markers previously published, 15.5 minutes (TAYLOR and ADELBERG 1960).

TABLE 7

Analysis of a cross involving JC-247, an Hfr isolated from the kinetic experiment (Figure 2)

Selected markers*		*pro*+(*his*+)	*met*+(*his*+)
Recombination frequency		6.0	0.2
Percent unselected male allele in selected recombinants	*pro*+	. .	48
	lac+	82	25
	mal−	0 (≤ 2.5)	30
	*str*R	0 (≤ 2.5)	28
	xyl−	0 (≤ 2.5)	40
	met+	0 (≤ 2.5)	. .
	♂	0 (≤ 2.5)	50

* Recombinant selections were made from a standard 120-minute mating of JC-247 male × AB-211 female. Forty colonies of each class were scored for unselected markers. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture.

The next genetic question posed by strain D2 concerns linkage between two markers separated by a sex factor. D2 may be viewed as a JC-12a type male with a sex factor interposed between *arg* and *TL*. Since these two markers are linked in the parent JC-12a, we may ask if they remain linked when situated on opposite sides of a sex factor. Presumptive evidence on this point can be obtained from the interrupted mating experiment already discussed. Reference to Figure 2 will show that, although linkage between *arg*+ and sex factor is suggested by the rise in the proportion of *arg*+ recombinants which are male, there is no such suggestion of linkage between *arg*+ and *TL*+, i.e., the proportion of TL+ in arg+ recombinants remains constant at a very low level (approximately five percent).

An additional experiment has been done to substantiate this result and is summarized in Table 8. In this experiment linkage between *arg* and the sex factor is again indicated by the fact that a change in selection from a distal marker (*mal*+) to a more proximal marker (*arg*+) increases the proportion of males among the recombinants. Change in selection from *mal*+ to *arg*+ does not, however, increase the proportion of *TL*+ recombinants; this proportion remains

TABLE 8

Cross between D2a^3 *male and AB-331 female to determine if linkage exists between* arg *and* TL *in conjugating cells of the double male*

Unselected marker	Selected marker†	Percent of recombinants, selected at various times after the beginning of mating, which possess the male allele of the unselected marker: 60 min.	90 min.	120 min.	120 min.*
sf (♂)	*mal*+	28	35	42 (≤ 1)‡	51 (≤ 1)
	arg+	56	60	75 (≤ 1)	72 (2)
TL+	*mal*+	4	6	4 (4)	26 (15)
	arg+	5	9	4 (16)	38 (39)

* Mating uninterrupted.
† Recombinant selections were made from a mating mixture composed of 20 female cells to every one male cell. Mating was interrupted with phage except where indicated. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture. Eighty colonies of each class were scored for unselected markers.
‡ Numbers in parentheses are data from a control cross of AB-1332 male × AB-331 female performed by A. L. TAYLOR under the same conditions as the cross involving D2a^3. AB-1332 is a recombinant derived from JC-12a.

constant even though enough time is allowed for *TL* to enter, as is seen from the control cross of another male related to JC-12a.

Unfortunately the opposite conclusion may be drawn from the data given in Table 8 for the uninterrupted mating between D2a^3 and AB-331. The plating at 120 minutes without interruption of mating indicates linkage between *arg* and *TL*. Apparently, couples remaining intact after 120 minutes may effect the belated transfer of *TL*. Since pair formation on the selective plates does not occur at the dilutions employed, the reason for this belated transfer is not known.

The genetic stability of D2 has been checked by examining 1600 isolated clones derived from D2a^3 for *xyl*$^-$ segregants. These colonies, present on 17 plates, were replicated onto eosin-methylene blue minimal medium (LEDERBERG, 1950) containing xylose as the only utilizable carbon source. By comparison with appropriate controls, it was found that all colonies were able to utilize xylose for growth.

Because D2 is somewhat of a genetic curiosity, some of its physiological properties were also determined. In complex broth at 30°C strains D2 and D2a^3 grew with generation times of 47 and 48 minutes respectively while JC-158a doubled every 52 minutes and JC-12a, every 45 minutes. At 37°C in the same medium all four strains grew with generation times of 29 minutes. Under these conditions then, the double male grows at the same rate as its parents.

Several other physiological parameters of D2a^3 were measured and are listed in Table 9 as compared with those of the parent strains. Exponentially growing cultures in complex broth at 30°C were harvested by centrifugation (4°C) about one generation before the onset of oxygen limitation. The cells were washed once in M/50 phosphate buffer at pH 7.0 and then resuspended in a small amount of distilled water. Aliquots of this suspension were used for

TABLE 9

*Determination of the amount of DNA in the nuclear bodies of D2a^3 and its parents**

	JC-158a	JC-12a	D2a^3
Generation time (minutes)	49	63	55
Dry weight (mg./ml. culture)	0.271	0.234	0.274
Percent dry weight as protein	52.0	46.8	50.0
μg. DNA per 100 mg. protein	7.34	7.26	7.26
Viable cells/ml. ($\times 10^{-8}$)	4.6 ± 0.5	5.9 ± 0.5	4.6 ± 0.3
Visible cells/ml. ($\times 10^{-8}$)	3.8 ± 0.2	3.5 ± 0.4	4.7 ± 0.0
Average amount of DNA/cell			
μg. $\times 10^8$/viable cell	2.2 ± 0.2	1.3 ± 0.1	2.1 ± 0.1
μg. $\times 10^8$/visible cell	2.7 ± 0.1	2.2 ± 0.2	2.1 ± 0.0
Average number of nuclear bodies per visible cell	1.86	1.93	2.00
Average amount of DNA/nuclear body			
μg. $\times 10^8$ based on viable cell count	1.2 ± 0.1	0.7 ± 0.1	1.1 ± 0.1
μg. $\times 10^8$ based on visible cell count	1.4 ± 0.1	1.2 ± 0.1	1.1 ± 0.0
average of these two	1.3	0.9	1.1

* Exponentially growing cultures at the same optical density were used for the determinations. Cells were grown in complex broth at 30°C. The error represents the deviation of the various values from their average.

determination of dry weight, protein and DNA. Just previous to harvesting, samples of the cultures were taken for viable and total cell count and smears were made of a 1/2 dilution in M/50 phosphate buffer at pH 7.0 for the counting of nuclear bodies. The three strains were found to possess the same ratio of DNA to protein and to contain on the average about the same amount of DNA in each nuclear body.

DISCUSSION

It has been possible, by crossing one Hfr by another, to prepare a male strain of *Escherichia coli* K-12 which possesses two sex factors attached at different points on the bacterial linkage group. This double male, although physiologically similar to its parents, appears to be genetically unique. It has two points of origin of chromosome transfer and expresses them both in the same clone. At present, it cannot be decided whether the two points of origin have the same probability of being expressed.

The genetic uniqueness of the double male in possessing two sex factors and two corresponding points of origin cannot be explained by viewing the strain as a stable association of parent cells. Purification by micromanipulation of single cells under the microscope demonstrates that both sex factors lie within a single cell. The strain must then be pictured according to the present formal understanding of the bacterial chromosome. Several models are suggested in Figure 3.

It is evident that D2 is not totally diploid as is pictured in the first model. Analysis of the average DNA content per nuclear body showed about the same value for the double male as for its parent strains. Another test of diploidy may be applied to this model. The marker *thi*$^-$ has been determined to be recessive to *thi*$^+$ (CLARK, unpublished) by heterozygosity tests similar to those used by LEDERBERG (1949). A heterozygous strain (*thi*$^+$/*thi*$^-$) would, therefore, grow independently of thiamin. This is the situation which would have been expected in a diploid resulting from a cross of JC-12a by JC-158a. Since the double male requires thiamin for growth, it may be that the double male is hemizygous *thi*$^-$ and consequently is haploid. The expression of the presumably recessive marker *ade*$^-$ in the double male lends support to this conclusion.

The argument based on expression of recessive markers in the double male may also be used against the idea that the double male is a persistent dikaryon (Model 2). A more powerful argument, however, is based on the finding that the two sex factors of the double male lie on the same linkage group. Consequently the double male must be a haploid monokaryotic strain.

Now we may answer the question posed in the introduction: does the attachment of the second sex factor between the origin and the terminus of the linkage group of an Hfr cause a second break with the production of two linkage groups? An alternative hypothesis would be that only one break may occur at a time and that each break is reversible. Thus a heterogeneous male population might be established (Model 3 of Figure 3). This hypothesis predicts that linkage between *arg* and *TL* would be retained in those male cells which inject *mal* as an early marker. It was shown, however, that there is no linkage between *arg* and *TL* in

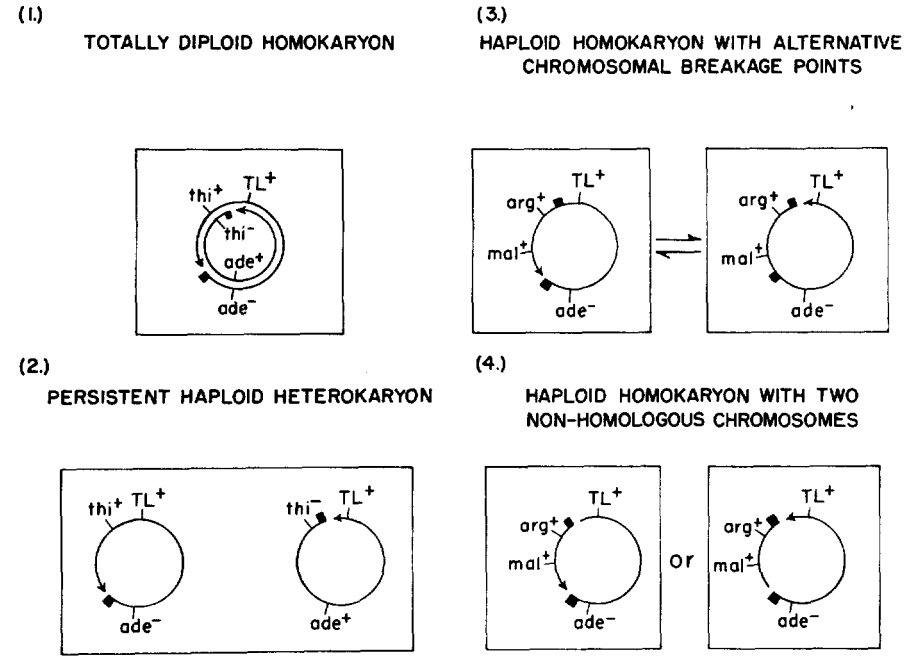

FIGURE 3.—Four hypothetical models to explain the behavior of the double male. Each box represents a cell and a circular area represents a nucleus. The circle itself represents the bacterial linkage group or its physical counterpart, the chromosome. Two concentric circles represent a diploid nucleus. Definitions of other symbols are to be found in the legend to Figure 1.

such males. Consequently, there must be a break between *arg* and *TL* (presumably at the sex factor which lies between them) and in conjugating cells of the double male which are capable of transferring chromosomal markers, there must be two linkage groups (Model 4 Figure 3). Any given cell, however, appears to transfer one or the other linkage group but not both (if we ignore the belated transfer of the second linkage group after 120 minutes in uninterrupted matings).

The genetic material of Hfr cells may exist in any one of three possible conditions: (1) normal vegetative replication, (2) transfer during conjugation, and (3) a condition of inactivity, in which there is neither replication nor transfer (i.e., in the F^- phenocopy). The experiments above have been concerned with genetic material of the double male in condition (2) exclusively. No information is as yet available on the number of linkage groups which the double male would possess if its genetic material were in conditions (1) or (3). An experiment with another strain, however, leads to the expectation that only one linkage group would be present in condition (3).

TAYLOR and ADELBERG (1961) have crossed two Hfr strains derived from a single cross as Hfr recombinants. Hence the sex factor in each is attached at the

same chromosomal site. One Hfr strain was converted to an F^- phenocopy by growth into the stationary phase; consequently this strain acted exclusively as a recipient in the cross. Linkage was then tested between markers close to the point of origin and close to the terminus of the injected linkage group. If the recipient chromosome had possessed an open structure, no linkage would have been observed. Strong linkage was observed, however; and the authors concluded that the chromosome of an Hfr strain which is acting as a recipient in conjugation is a closed structure.

It is particularly important to ascertain the number of linkage groups present in vegetative cells of the double male. If one assumes that a bacterial linkage group represents a physical structure along which genes are arrayed (i.e., a chromosome), the double male might possess two chromosomes during vegetative growth. Without a mitotic mechanism of some sort, the double male might often fail to achieve equipartition of its genetic material at division, and the probability of the production of nonviable cells would be high. In fact, however, the growth of the double male is exponential and its growth rate is not much different from the growth rates of its parents under several conditions. In addition it was determined (Table 9) that during exponential growth the number of viable cells of the double male equals the number of cells microscopically visible. Consequently it seems probable that the double male possesses only one chromosome during vegetative growth, although rigorous proof of this assumption will require further genetic analysis.

To summarize, study of a double male has shown the following:

(1) Two sex factors may coexist indefinitely in the same cell if both are attached stably to the chromosome; and

(2) A strain carrying two stably attached sex factors transfers genetic material to recipients in the form of two independent non-homologous linkage groups. Any given cell appears to transfer one or the other linkage group, but not both.

SUMMARY

From a cross of two Hfr strains of *Escherichia coli* K-12 a haploid monokaryotic Hfr has been obtained which contains two chromosomally-linked sex factors. This strain is viable and stable and transfers genetic material to recipients in the form of two nonhomologous linkage groups.

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LITERATURE CITED

ADELBERG, E. A., and S. N. BURNS, 1960 Genetic variation in the sex factor of *Escherichia coli*. J. Bacteriol. **79**: 321–330.

BURTON, K., 1956 A study of conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**: 315–323.

CAVALLI-SFORZA, L. L., J. LEDERBERG, and E. M. LEDERBERG, 1953 An infective factor controlling sex compatibility in *Bacterium coli*. J. Gen. Microbiol. **8**: 89–103.

CLARK, A. J., 1961 Preparation of a strain of *Escherichia coli* K-12 possessing two linkage groups. Bacteriol. Proc. 1961: 98.

HAYES, W., 1953 The mechanism of genetic recombination in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol. **18**: 75–93.

HAYES, W., 1957 The kinetics of the mating process in *Escherichia coli*. J. Gen. Microbiol. **16**: 97–119.

JACOB, F., and E. L. WOLLMAN, 1956 Recombination génétique et mutants de fertilité chez *Escherichia coli*. Compt. rend. **242**: 303–306.

JACOB, F., and E. L. WOLLMAN, 1957 Analyse des groupes de liaison génétique de différentes souches donatrices d'*Escherichia coli* K-12. Compt. rend. **245**: 1840–1843.

1958 Sur les processus de conjugaison et de recombinaison génétique chez *Escherichia coli*. IV. Prophages inductibles et mesures des segments génétiques transférés au cours de la conjugaison. Ann. Inst. Pasteur **95**: 497–519.

KELLENBERGER, E., 1960 The physical state of the bacterial nucleus. Symposium Soc. Gen. Microbiol. **10**: 39–66.

LEDERBERG, J., 1949 Aberrant heterozygotes in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **41**: 359–364.

1950 Isolation and characterization of biochemical mutants of bacteria. Methods in Med. Res. **3**: 5–22.

1954 A simple method for isolating individual microbes. J. Bacteriol. **68**: 258.

1958 Extranuclear transmission of the F compatibility factor in *E. coli*. Abstr. Communs., 7th Intern. Congr. Microbiol., Stockholm, 59–60.

LEDERBERG, J., L. L. CAVALLI, and E. M. LEDERBERG, 1952 Sex compatibility in *Escherichia coli*. Genetics **37**: 720–730.

LEDERBERG, J., and E. M. LEDERBERG, 1952 Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**: 399–406.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

MONOD, J., G. COHEN-BAZIRE, and M. COHN, 1951 Sur la biosynthese de la β-galactosidase (lactase) chez *Escherichia coli*. La spécificité de l'induction. Biochim. Biophys. Acta **7**: 585–599.

ROBINOW, C. F., 1942 A study of the nuclear apparatus of bacteria. Proc. Roy. Soc. London B **130**: 299–324.

SCHNEIDER, W. C., 1945 Extracts of cellular compounds. J. Biol. Chem. **161**: 293–303.

TAYLOR, A. L., 1961 Genetic studies with very high frequency males of *Escherichia coli*. Ph.D. dissertation, University of California, Berkeley.

TAYLOR, A. L., and E. A. ADELBERG, 1960 Linkage analysis with very high frequency males of *Escherichia coli*. Genetics **45**: 1233–1243.

1961 Evidence for a closed linkage group in Hfr males of *Escherichia coli* K-12. Biophys. Biochem. Res. Comm. **5**: 400–404.

WOLLMAN, E. L., and F. JACOB, 1958 Sur le déterminisme génétique des types sexuels chez *Escherichia coli* K-12. Compt. Rend. **247**: 536–539.

WOLLMAN, E. L., F. JACOB, and W. HAYES, 1956 Conjugation and genetic recombination in *Escherichia coli* K-12. Cold Spring Harbor Symp. Quant. Biol. **21**: 141–162.