INCOMPATIBILITY OF INTEGRATED SEX FACTORS IN DOUBLE MALE STRAINS OF *ESCHERICHIA COLI**

ANTHONY R. KANEY

Dept. of Biology, Bryn Mawr College, Bryn Mawr, Pa.

AND

K. C. ATWOOD

Dept. of Human Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, N. Y.

Manuscript received September 2, 1971
Revised copy received October 4, 1971

ABSTRACT

Several strains of *Escherichia coli* K-12 harboring two F factors were isolated from Hfr × Hfr crosses. These strains were transiently capable of initiating chromosome transfer from two separate points of origin, and of transferring two different sex factors as integrated chromosomal markers. Each strain tested invariably reverted to a simple Hfr by loss of one of the inherited F factors. The F factor persisting in the revertant was, in nearly every case, that which had been inherited from the recipient Hfr parent.

THE incompatibility occurring with F and F′ factors transferred to cells of *Escherichia coli* K-12 already harboring integrated (Hfr) or autonomous (F^+, F′) F factors has been previously reported (SCAIFE and GROSS 1962; MAAS 1963; CUZIN and JACOB 1967; DUBNAU and MAAS 1968; MAAS and GOLDSCHMIDT 1969). When the recipient strain is Hfr, the incoming episome fails to replicate and is rapidly diluted out of the culture in subsequent divisions. When the recipient strain is F^+ or F′, the recombinant cells segregate two cell lines, each harboring only one of the autonomous factors. Rarely, a stable double male strain results from such crosses, harboring one integrated and one autonomous (CUZIN 1962; MAAS and GOLDSCHMIDT 1969) or two autonomous (CUZIN and JACOB 1967) F factors.

Suggested explanations for the phenomenon of incompatibility have been discussed by DUBNAU and MAAS (1968) and postulate, on the one hand, that an "immunity substance" analogous to the repressor elaborated by bacteriophage *lambda* in lysogenic cells (PTASHNE 1967) could be responsible. Alternatively, it may be necessary for F to be attached to a "maintenance site", presumably on the cell membrane, for replication. The latter hypothesis assumes that there is only one maintenance site per genome. If it is occupied by a resident F factor, then any superinfecting factor cannot be replicated. The rare appearance of

* This work was partially supported by National Science Foundation grant GB296.

double male strains such as those mentioned above might indicate the presence of some mutational change leading to non-production of or insensitivity to repressor, or synthesis of additional maintenance sites. The priority of an integrated F factor to an autonomous one has led to the suggestion that an autonomous factor can escape the inhibition by itself becoming integrated into the chromosome (CAMPBELL 1969). The isolation of apparently stable strains harboring two integrated F factors (CLARK 1963; DEVRIES and MAAS 1971) would seem to verify this possibility. However, this paper reports the isolation of several double male strains initially harboring two integrated sex factors, all of which were invariably unstable, reverting to simple Hfr strains by the loss of one factor. This finding suggests that the presence of multiple F factors, even if integrated, is an anomalous condition in some strains.

MATERIALS AND METHODS

Bacterial strains: All bacterial strains used in this investigation are derivatives of *Escherichia coli* K-12, and are described in Table 1. Hfr strains K22, K12, and K27 were derived from strains AB492, AB257, and AB2271 respectively, which were kindly supplied by Dr. E. A. ADELBERG.

Media and Mating Techniques: These are generally as described previously (YU, KANEY and ATWOOD 1965). The crosses for isolation of double male strains were performed as follows: The Hfr strain to be used as the recipient was converted to F^- phenocopy by growing in stationary phase with aeration for 18–20 hr. This culture and an exponentially growing culture of the donor were diluted in broth to contain about 2×10^8 cells per ml, and were chilled on ice. When chilled, 0.5 ml of the donor culture was mixed with 4.5 ml of the recipient culture and the mixture was filtered through a Millipore membrane filter with pore size of 0.45 microns using the technique of MATNEY and ASCHENBACH (1962). The filter was then placed on a plate of nutrient agar and incubated at 37°C for 120 min. The filter was then removed, and the cells were washed off and plated on selective media.

RESULTS

A cross was performed between Hfr strains K22 and K12 as described in METHODS, using K22 as donor and K12 as recipient. A diagram of the cross is shown in Figure 1a. Selection was for *pro*$^+$ *pyrD*$^+$ *his*$^+$ *gua*$^+$ recombinants, which were obtained with a frequency of 4.35×10^{-3} per input donor. Four hundred recombinants were first replica-plated to the selective medium, and then

TABLE 1

		Bacterial strains
Strain	Sex	Genetic markers*
K22	Hfr	*arg*$^-$ *thi*$^-$ *thy*$^-$ *ade*$^-$ *pro*$^-$ *pyrD*$^-$ *ilv*$^-$ *mtl*$^-$
K12	Hfr	*met*$^-$ *gua*$^-$ *his*$^-$ *ilv*$^-$ *mtl*$^-$ *lac*$^-$
K27	Hfr	*pro*$^-$ *gua*$^-$ *his*$^-$ *pyrD*$^-$ *thr*$^-$ *lac*$^-$ *str*^{R}
K10	F$^-$	*his*$^-$ *pro*$^-$ *lac*$^-$ *str*^{R}
K49	F$^-$	*his*$^-$ *pro*$^-$ *trp*$^-$ *thr*$^-$ *lac*$^-$ *str*^{R}
K34	F$^-$	*met*$^-$ *arg*$^-$ *gua*$^-$ *pyrD*$^-$ *ilv*$^-$ *str*^{R}

* Gene symbols described by TAYLOR and THOMAN (1964).

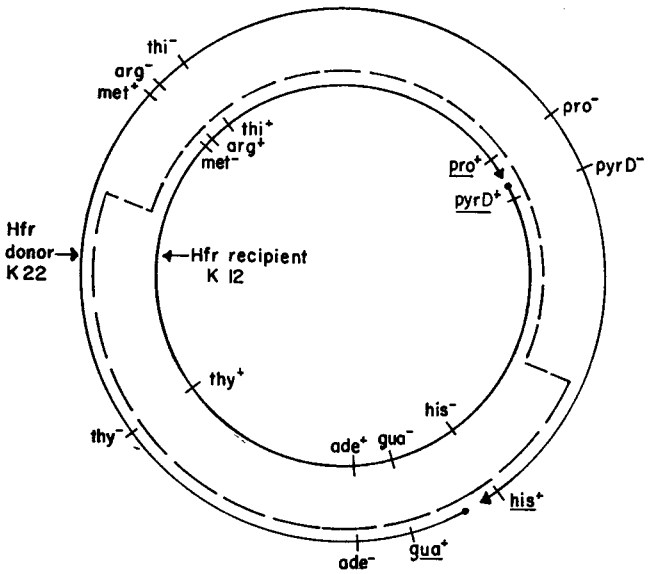
to master plates of nutrient agar, after which they were tested for inheritance of the unselected markers *ade* and *thy*. All 400 isolates were *ade*$^-$, while 122 were *thy*$^+$ and 278 were *thy*$^-$. All isolates were then replica-plated to media selective for *his*$^+$ and for *pro*$^+$ recombinants on which had been spread a lawn of F$^-$ strain K10. The rationale for this test is as follows: only those isolates which had inherited the origins from both parental strains should give rise to patches of recombinant growth on both selective media. Those inheriting the origin of only one of the parents should give rise to growth on one or the other, but not both plates. In practice, a control of Hfr K22 gave rise to recombinant growth on both media, but the growth on the medium selective for *pro*$^+$ recombinants was very sparse and appeared after 48 hr or more of incubation. In this test, 48 of the 400 isolates gave rise to early appearing, heavy patches of recombinant growth on both media. Each of the 48, all of which had the genotype *met*$^-$ *thy*$^-$ *ade*$^-$, was purified by single colony isolation from a streaked plate and the test was repeated. At this time, subclones of only 11 of the 48 isolates still produced recombinants on both media, and after a further purification, subclones of only 3 of the 11 retained the ability. Subclones of the remaining 45 isolates were capable of producing recombinant growth on the medium selective for *pro*$^+$, but had apparently lost the ability to produce *his*$^+$ recombinants. One of the three remaining presumptive double male isolates, labelled type K2212, was used as donor in an interrupted mating with F$^-$ strain K49. Time of entry was determined for the four markers *his*$^+$, *trp*$^+$, *pro*$^+$, and *thr*$^+$. The results of this mating are shown in Figure 2. As can be seen, K2212 shows transfer kinetics characteristic of a donor strain having two different points of origin, one near the *his* locus and the other near *pro*. Lack of linkage between *his* and *pro* in K2212 was indicated by the finding that none of 100 *his*$^+$ and *pro*$^+$ recombinants from the 40 min sampling had also inherited the other marker. This is in accordance with the previous finding (CLARK 1963) that double male strains initiate transfer from only one point of origin at a time.

If this strain has two stably integrated sex factors, then all or most of the *his*$^+$ and *pro*$^+$ recombinants from the above mating should be F$^-$. To test this, 100 each of the *his*$^+$ and *pro*$^+$ recombinants from the 40 min sample were screened with the male-specific bacteriophage f2 (LOEB and ZINDER 1961). Although the phage gave zones of complete lysis in control spot tests with the parental Hfr strains, the tests were negative for all of the *his*$^+$ recombinants, and positive for only 3 of the *pro*$^+$ recombinants. These latter did not appear to be merodiploid, however, since they did not transfer *pro*$^+$ to recipient cells, nor did they give rise to *pro*$^-$ segregants.

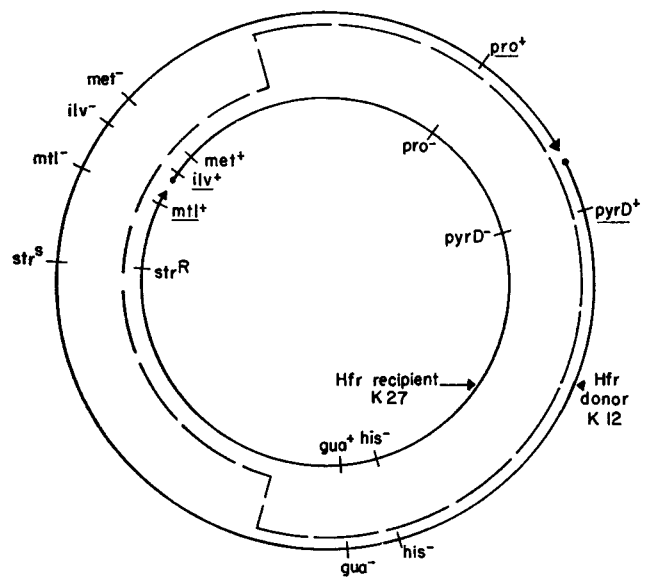
Finally, the two sex factors of K2212 were mapped as chromosomal markers by mating it with F$^-$ strain K34 for 90 min. Selection was for *gua*$^+$ and *pyrD*$^+$ recombinants, these markers being closely linked to the presumed sex factors. Fifty of each type of recombinant were then spot-tested with bacteriophage f2 to determine inheritance of the sex factor. The results of this experiment are shown in Table 2. One each of the *gua*$^+$ and *pyrD*$^+$ recombinants was subsequently mated with F$^-$ strain K49, and gave transfer kinetics which were virtually identi-

cal to those of parental Hfr strains K22 and K12, respectively. Thus, by the usual criteria, K2212 is a double male strain with two integrated sex factors.

In subsequent single colony purifications, it was found that K2212, as well as the other two isolates of this type in which double origin characteristics had persisted, invariably segregated a high proportion of clones incapable of early



A



B

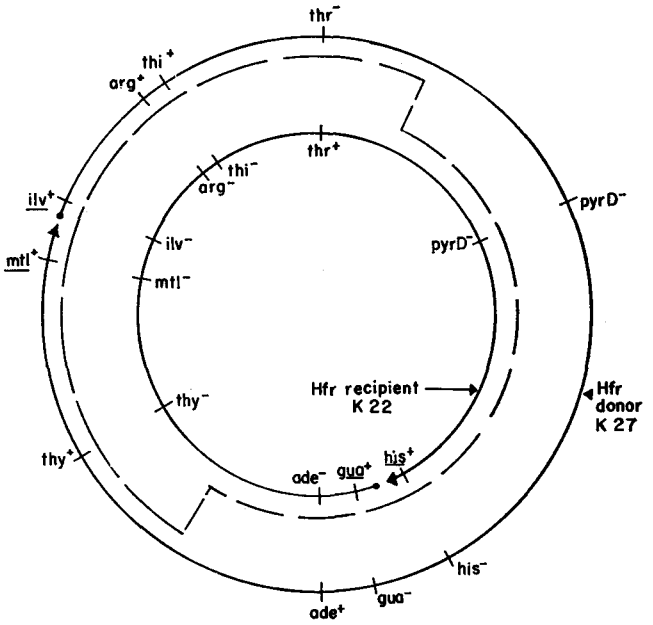


FIGURE 1.—Schematic representation of mating yielding double male strains of types (A) K2212, (B) K1227, and (C) K2722. Dotted line represents recombinant linkage group giving rise to double male with same genotype as strain described in text. Selected markers are underlined.

transfer of *his*+. The kinetics of this breakdown was not analyzed in great detail, however, it was determined that the ratio of double male to Hfr subclones decreased at each step in a series of single colony isolations. In one case, for example, the double male isolate was streaked on a plate of nutrient agar. One hundred or more subclones were then tested for donor characteristics by replication with wooden applicator sticks to plates of selective media spread with lawns of strain K10 as described previously. The ratio of double male to Hfr subclones was noted, and then the process was repeated by again streaking one of the double male subclones. After the first transfer, the ratio of double male to Hfr subclones was 37/63, after the second, 21/79, after the third, 3/97, and after the fourth transfer no double male clones were detected among 400 tested.

Ten of the Hfr segregants, when used as donors in interrupted matings, proved to have transfer kinetics which were virtually identical to those of parental Hfr

TABLE 2

Inheritance of sex factor by recombinants from cross K2212 × K34

Selected marker	Recombinant/donor	Percent recombinant ♂
pyrD+	0.080	41
gua+	0.0065	36

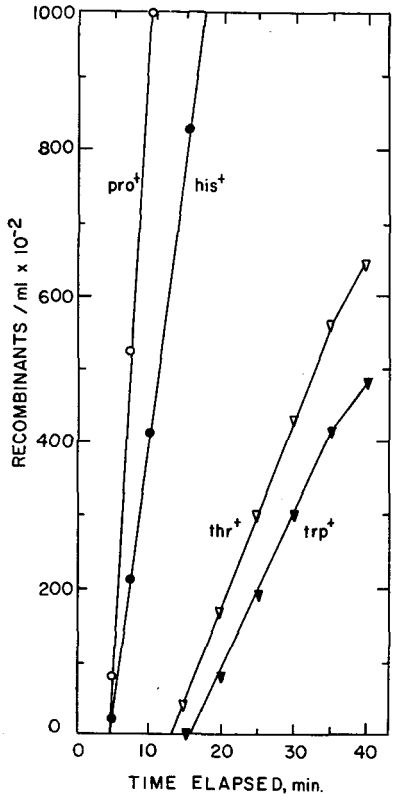


FIGURE 2.—Kinetics of recombinant formation in cross K2212 × K49.

K12, with *his*$^+$ being transferred only after 65 min. A test of 100 such *his*$^+$ recombinants with phage f2 revealed none that were male. Hence, it appeared that the 3 double male strains tested directly, and presumably the other 45 original isolates as well, rapidly segregated Hfr cells which had lost the sex factor inherited from K22. A further bit of evidence for this was the finding that, of 100 tested, 3 of the Hfr revertants of K2212 had become *his*$^-$. These revertants were phenocopied and used as recipients in a cross with Hfr K22, which transfers *his*$^+$ early and at high frequency. After mating for 120 min, the cells were plated on media selective for *his*$^+$ and *met*$^+$ recombinants. In each case, although *met*$^+$ recombinants were obtained with frequencies of about 1.6×10^{-4}, the frequency of *his*$^+$ recombinants was less than 1.0×10^{-5}. Since *met*$^+$ is transferred 50 min later than *his*$^+$ by this Hfr, these results strongly suggest that the 3 revertants contain deletions of part or all of the *his* locus. Significantly, no *his*$^+$ revertants have been found from these strains after an intensive search for them. These assumed deletions of the *his* locus could have been incurred at the loss of the closely linked K22 F factor.

The finding that every revertant had lost the sex factor inherited from the donor Hfr was of singular interest. It would appear that this was simply due to the inherently less stable insertion of the K22 sex factor, although no such insta-

TABLE 3

Revertant types produced by double male strains

Double male type	Donor	Recipient	Number tested	Number giving only revertants of type K22	K12	K27
K2212	K22	K12	48	0	48	..
K1222	K12	K22	40	39	1	..
K2122	K27	K22	26	25	..	1
K1227	K12	K27	13	..	0	13

bility had been previously apparent. To retest this finding in a preliminary way, the cross of K22 and K12 was repeated in reciprocal fashion, that is, using K22 as recipient and K12 as donor. A total of 83 recombinants were obtained capable of early transfer of both *his*$^+$ and *pro*$^+$. Forty of these isolates, labelled double male type K1222, were tested for the nature of their breakdown products by repeated streaking on nutrient agar plates and testing of single colony isolates with strain K10 on selective media as before. One hundred subclones were tested from each of the 40 isolates, and 39 of these yielded only revertants capable of early transfer of *his*$^+$ alone, while 1 yielded only revertants capable of early transfer of *pro*$^+$ alone. Interestingly, no isolates yielded revertants of both types within the sample tested. Thus, the phenomenon is apparently not strain specific, rather, there is a strong preferential loss of the F factor inherited from the donor parent.

Subsequently, two other types of double male strains were isolated, as shown in Figure 1b and 1c. The cross of K27 with K12 yielded 13 recombinants capable of early transfer of both *pro*$^+$ and *mtl*$^+$. These strains, all having the genotype *str*^{r} *gua*$^-$ *his*$^-$, were labelled double male type K2712. The cross of K22 with K27 gave 26 recombinants capable of early transfer of both *mtl*$^+$ and *his*$^+$. These strains, all having the genotype *ade*$^-$ *pyrD*$^-$ *thr*$^-$, were labelled double male type K2227. One isolate of each type showed typical double male characteristics by all the criteria used in testing type K2212. Again, however, all isolates reverted to simple Hfr strains very rapidly. With one exception, all revertants had lost the sex factor inherited from the Hfr donor. This data is summarized in Table 3.

It should also be noted that, in most cases, the reversion of the double male strains could be observed on a nutrient agar plate. A lawn or heavy streak of the double male gave rise, after 4–5 days, to numerous papillae, usually of a different color and/or texture from the underlying growth. These papillae, when tested, proved to be Hfr revertants which were otherwise identical to the double male in nutritional requirements, but which had lost the sex factor inherited from the donor parent.

DISCUSSION

The data presented demonstrate that the incompatibility between two F factors in the same cytoplasm cannot always be obviated by having both factors integrated into the chromosome. Over 400 strains were isolated which initially

showed double male characteristics, yet all rapidly reverted to the Hfr state. Furthermore, no stable derivatives of any of these strains could be found.

One of the previously isolated strains having two integrated F factors was also formed by mating two Hfr strains (CLARK 1963) and no instability was reported for this strain. However, in the case of double male strains isolated by mating an Hfr with an F′-*lac* strain (DEVRIES and MAAS 1971) some instances of instability were noted in that strains which initially appeared to have stable integration of the episome were subsequently found to segregate free F′-*lac* factors and *lac*$^-$ clones.

The incompatibility between an integrated and an autonomous F factor has been shown to result in the inhibition of replication of the autonomous factor (DUBNAU and MAAS 1968). It remains unclear, however, by what means such incompatibility between two integrated factors might operate.

The assumption that both parental F factors were initially integrated in the double male strains reported is strongly supported by several bits of evidence. Inheritance of donor markers distant from the origin suggests that the double males arose from the transfer of a complete chromosome, rather than F′ elements, from the donor. In addition, it was observed that no double male recombinants were obtained if the initial cross was stopped after only 60, rather than 120 min. The finding that the overwhelming majority of recombinants for proximal markers generated by the double males was F$^-$ also indicates integration of the F factors. Finally, the inheritance of maleness by recipients concomitant with markers closely linked to the insertion sites of the double male factors was clearly shown.

Tentative interpretations of the data given cannot be considered without taking into account the probable but uncertain role of integrated F in chromosome replication. Although there has been convincing evidence for both F-initiated (NAGATA 1963; VIELMATTER, MESSER and SCHÜTTE 1968) and F-independent (BERG and CARO 1967; CERDÁ-OLMEDO, HANAWALT and GUEROLA 1968) replication, the question has not been settled. It appears that F can serve as one, but not the only possible site for initiation of replication, and that there are strain-specific variations in this pattern. If F plays no role in chromosome replication, there is no *a priori* reason why multiple integrated sex factors would not be tolerated. If it is involved, however, one can envision possible disorganizations of replication resulting from multiple initiation or termination sites in double males. These considerations deal with the effects of the two integrated F factors on the chromosome, but fail to take into account any possible effects that they have upon one another directly. The specificity of F factor loss in these strains strongly suggests such an interaction, but ideas as to the nature of the interaction must remain for the present, pure speculation. It is obvious that further work is necessary to clarify the problem of F incompatibility and its relation to chromosome replication.

LITERATURE CITED

BERG, C. M. and L. G. CARO, 1967 Chromosome replication in *Escherichia coli*. I. Lack of influence of the integrated F factor. J. Mol. Biol. **29**: 419–434.

CAMPBELL, A. M., 1969 *Episomes*. Harper and Row, New York.

CERDÁ-OLMEDO, E., P. C. HANAWALT and N. GUEROLA, 1968 Mutagenesis of the replication point by nitrosoguanidine: map and pattern of replication of the *Escherichia coli* chromosome. J. Mol. Biol. **33**: 705–722.

CLARK, A. J., 1963 Genetic analysis of a "double male" strain of *Escherichia coli* K-12. Genetics **48**: 105–120.

CUZIN, F., 1962 Multiplication autonome de l'episome sexuel d'*Escherichia coli* K-12 dans une souche Hfr. Compt. Rend. **254**: 4211–4213.

CUZIN, F. and F. JACOB, 1967 Association stable de deux episomes F differents dans un clone de *Escherichia coli*. Ann. Inst. Pasteur **113**: 145–155.

DEVRIES, J. K. and W. K. MAAS, 1971 Chromosomal integration of F′ factors in recombination-deficient Hfr strains of *Escherichia coli*. J. Bacteriol. **106**: 150–156.

DUBNAU, E. and W. K. MAAS, 1968 Inhibition of replication of an F-lac episome in Hfr cells of *Escherichia coli*. J. Bacteriol. **95**: 531–539.

LOEB, T. and N. ZINDER, 1961 A bacteriophage containing RNA. Proc. Natl. Acad. Sci. U.S.A. **47**: 282–289.

MAAS, R., 1963 Exclusion of an F-*lac* episome by an Hfr gene. Proc. Natl. Acad. Sci. U.S.A. **50**: 1051–1055.

MAAS, W. K. and A. D. GOLDSCHMIDT, 1969 A mutant of *Escherichia coli* permitting replication of two F factors. Proc. Natl. Acad. Sci. U.S.A. **62**: 873–880.

MATNEY, T. S. and N. E. ASCHENBACH, 1962 New uses for membrane filters III: Bacterial mating procedure. J. Bacteriol. **84**: 874–875.

NAGATA, T., 1963 The molecular synchrony and sequential replication of DNA in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **49**: 551–559.

PTASHNE, M., 1967 Isolation of the *lambda* phage repressor. Proc. Natl. Acad. Sci. U.S.A. **57**: 306–313.

SCAIFE, J. and J. D. GROSS, 1962 Inhibition of multiplication of an F-*lac* factor in Hfr cells of *Escherichia coli*. Biochem. Biophys. Res. Comm. **7**: 403–407.

TAYLOR, A. L. and M. S. THOMAN, 1964 The genetic map of *Escherichia coli* K-12. Genetics **50**: 659–677.

VIELMETTER, W., W. MESSER and A. SCHÜTTE, 1968 Growth direction and segregation of the *E. coli* chromosome. Cold Spr. Harb. Symp. Quant. Biol. **33**: 585–598.

YU, M. T., A. R. KANEY and K. C. ATWOOD, 1965 Genetic mapping of fructose 1–6 diphosphatase in *E. coli*. J. Bacteriol. **90**: 1150–1153.