# ASSOCIATION BETWEEN COLICINOGENIC AND FERTILITY FACTORS[1]

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COLICINOGENIC factors (*col* factors) are transferred among different Hfr or $F^+$ and $F^-$ strains of *Escherichia coli* K-12 as extrachromosomal particles (FREDERICQ and BETZ-BAREAU 1953; NAGEL DE ZWAIG, ANTÓN, and PUIG 1962; CLOWES 1963). While some factors, like $colE_2$ and $colE_1$, do not affect the fertility of donor strains, others, for example colB or colI, have been reported either to cause fertility or to interfere with fertility in *Salmonella typhimurium* or *E. coli* strains (OZEKI, STOCKER, and SMITH 1962; PUIG and NAGEL DE ZWAIG 1964; NAGEL DE ZWAIG and PUIG 1964).

A colicinogenic plasmid colV colI derived from strain *E. coli* K94 from P. FREDERICQ, which controls the synthesis of colicins V and I, is transferred with very high efficiency in mating (NAGEL DE ZWAIG and ANTÓN 1964). Several observations indicate that this plasmid possesses some properties characteristic of fertility factors: (a) all $F^-$ cells acquiring colV colI become sensitive to the male specific phage MS2, (b) the presence of the colV colI plasmid in an $F^-$ cell confers on it the ability to conjugate and promotes not only its own efficient transfer but also, to a smaller degree, that of the bacterial chromosome; (c) the colV colI plasmid interferes with chromosomal transfer by some Hfr or $F^+$ strains and with the expression or persistence of F-*lac* or F-*gal* factors (NAGEL DE ZWAIG and ANTÓN 1964). Similar observations were made on $F_v$colV factor by KAHN and HELINSKI (1964).

In view of these observations the question may be posed whether the F-like properties displayed by the colV colI factor reside in the genomes of the colicinogenic determinants, as appears to be the case for colI (OZEKI *et al.* 1963), whose fertility effect has not been separated from the colicinogenic property, or in an F factor associated with the col determinants, as reported by FREDERICQ (1963b) for colV or colB.

The present report presents evidence that the fertility conferred on the host bacteria by colV colI can be separated from the colicinogenic determinants. Some additional observations on the properties of bacteria carrying colV colI are also presented.

## MATERIALS AND METHODS

*Media:* The media used were: LB broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 liter

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distilled water; pH 7); LB agar (1% agar); Difco Penassay broth; Difco Penassay agar (Difco Penassay broth + 1.5% agar); minimal agar (10.5 g $K_2HPO_4$, 4.5 g $KH_2PO_4$, 1.0 g $(NH_4)_2SO_4$, 0.47 g Na citrate, 50 mg $MgSO_4$, 15 g Bacto agar, 1 liter distilled water; glucose added to a final concentration of 0.5%) supplemented with different amino acids or thiamine at final concentrations of 20 μg/ml and 2 μg/ml respectively. Streptomycin, when required, was used at a concentration of 200 μg/ml.

*Bacterial and phage strains:* The bacterial strains employed are listed in Table 1. Some additional strains derived from those listed in Table 1 are described in the text. Strains resistant to colicins V and I are designated as /V,I.

Phages P1 and MS2 were from DR. LURIA's collection; f1 was received from DR. N. D. ZINDER's laboratory and Qβ from DR. S. SPIEGELMAN.

For details of conditions of mating and transfer of col factors see NAGEL DE ZWAIG *et al.* (1962). The frequency of chromosomal recombinants or of col+ $str^r$ cells expresses the ratio of the number of recombinants or col+ $str^r$ cells to the initial number of donor bacteria.

For prolonged mating, donor and recipient bacteria were mixed in a 1:1 proportion and three successive transfers in fresh broth (1/10 dilution) were made at intervals.

Colicin I production was determined by using an indicator strain resistant to colicin V and sensitive to colicin I (NAGEL DE ZWAIG and ANTÓN 1964). Colicin V production was tested using an indicator strain sensitive to both colicins, since the inhibition zone produced by colicin V is much larger than that of colicin I and easily distinguishable.

For acridine orange treatment the method of HIROTA (1960) was employed. When necessary, a second transfer of the strain in the medium with acridine orange was performed.

For nitrosoguanidine mutagenesis the method of ADELBERG *et al.* (1965) was followed.

Strains resistant to phage MS2 (=MS2$^r$) were isolated after three successive cycles of growth in LB broth in the presence of a high multiplicity of phage. Sensitivity or resistance to male specific phages was determined by spot tests.

*$P^{32}$ decay experiment:* An overnight culture of donor bacteria grown in low phosphate medium (~ 5 mg P/liter) was transferred to the same medium plus $P^{32}$ (specific radioactivity ~ 100 mc/mg P) and incubated to reach a titer of about $4 \times 10^8$ bacteria/ml, when about 3 to 4 generations had elapsed. Then the bacteria were mated with recipient cells grown in Penassay broth in the proportion of ten donors to one recipient cell. Unlabeled phosphate buffer pH 7.0 was added to the mating mixture to give a final concentration of 0.05 M. Samples were taken at different times, diluted in protective medium (minimal medium + 12% glycerol), distributed in small tubes, frozen in alcohol and dry ice, and stored at —20°C. Samples were thawed and tested at the desired times.

## RESULTS

### *Properties conferred on the host cell by the colV colI factor. Identification of*

TABLE 1

*Bacterial strains*

| Strain | Relevant characteristics |
|---|---|
| *E. coli* K94 | Prototroph, $str^s$ (colV colI) |
| *E. coli* K-12—AB1122 | *pro⁻arg⁻his⁻thi⁻lac⁻* $str^s$ F⁻ |
| *E. coli* K-12—AB1157 | *thr⁻leu⁻thi⁻pro⁻arg⁻his⁻* $str^r$ F⁻ |
| *E. coli* K-12—C600 | *thr⁻leu⁻thi⁻str-s* or $str^r$ F⁻ |
| *E. coli* K-12—PA309 | *thr⁻leu⁻thi⁻his⁻arg⁻try⁻* $str^r$ F⁻ |
| *Shigella dysenteriae* 16 | Prototroph, $str^r$ |

Symbols: *thr*=threonine; *leu*=leucine; *try*=tryptophan; *his*=histidine; *arg*=arginine; *met*=methionine; *pro*=proline; *thi*=thiamine; *str*=resistance or sensitivity to streptomycin; *lac*=fermentation of lactose.

*the type of colicin I produced:* There are several strains known to produce colicin I. The colI factor of a strain carrying colV colI was compared by immunity tests with the colI factor derived from *Shigella sonnei* strain P9 and the colI factor derived from *E. coli* strain CA 53. The presence of a colV colI factor in an otherwise sensitive strain confers complete immunity to the action of colicin $I_a$ derived from CA 53 and partial immunity to colicin $I_b$, derived from P9. The sensitivity of strain C600 (colV colI) to colicin $I_b$, measured from the number of survivors, is much lower than that of the original noncolicinogenic strain. These observations suggest that the colI factor that is part of colV colI may be identical to $colI_a$ and different from $colI_b$. STOCKER (1965) also found differences in the immunity conferred by $colI_a$ and $colI_b$ to the two colicins.

The colicin I produced by a strain carrying colV colI is temperature sensitive, like colicins $I_a$ and $I_b$.

*Sensitivity to male phages:* An $F^-$ cell receiving a colV colI factor acquires sensitivity to the male specific RNA phages MS2 and $Q\beta$ and also to the male specific DNA phage f1.

*Restriction on phage P1 growth:* Phage P1 is adsorbed normally (in calcium supplemented broth) by a strain carrying a colV colI factor and has the same transmission as on the noncolicinogenic strains, as measured by plating of infective centers on *E. coli* C600 or *Shigella dysenteriae* 16. However, the plaque count on strains carrying colV colI is lower and more variable than on noncolicinogenic bacteria. This is probably due to the much lower burst size of P1 from bacteria carrying colV colI than from noncolicinogenic bacteria (five instead of 120). The phage that comes out plates again with lower efficiency on colicinogenic bacteria than on noncolicinogenic ones. A supervirulent mutant of P1 was found, by spot tests, to be equally restricted in strains carrying colV colI. The nature of this effect on P1 multiplication is not known.

*Presence of pili:* Electron microscopic examination of strains C600 (colV colI) and AB1122 (colV colI) showed that both strains have F-type pili, that is, pili that specifically adsorb phage MS2 (BRINTON *et al.* 1964). When F-pili were present, there were one or two visible per bacterium. No F-pili were observed on either one of the $F^-$ strains C600 and AB1122.

*Association with a fertility determinant:* Two different approaches were followed in order to determine if the F properties of the colV colI factor were distinct from the colicinogenic property: (1) A $P^{32}$ decay suicide experiment aimed at achieving separation of the components factors of the colV colI plasmid. (2) Isolation and study of colicinogenic strains resistant to male phages.

*$P^{32}$ decay suicide experiment:* Highly labeled bacteria of strain AB1122 (colV colI) *$str^s$* were mated with unlabeled recipient AB1157/V,I *$str^r$*, in the proportion of ten donors to one recipient cell. At 5, 10, and 20 min after the onset of mating, samples were taken, frozen, and stored. At zero time and on successive days samples were thawed and plated on agar with streptomycin to select for recipient type cells. The colonies were tested for production of colicin V and colicin I on different plates and the proportion of colicinogenic cells was measured. The results showed that the ability to produce colicin V or colicin I was sensitive to

$P^{32}$ decay when the bacteria were frozen about 5 minutes after mating, but that by 20 minutes the colicinogenic property had become fully stabilized against decay.

A total of 97 colicin-producing colonies corresponding to the 5 minute sample, and 70 such colonies from the 10 minute sample, were picked from plates prepared between the 5th and the 14th days of storage and were retested for colicin production and for sensitity to phage MS2. All colicinogenic isolates produced both colicins and were sensitive to MS2. One exceptional colony was found which on the original plate had produced some colicin V, but, on retesting, proved to be noncolicinogenic. When this isolate was restreaked, the resulting colonies were found to be sensitive to phage MS2 but produced no colicin. This strain was called $AB1157^X$ and was studied in some detail.

*Properties of the plasmid carried by strain $AB1157^X$:* Cells of strain $AB1157^X$ were mixed with cells C600 $F^-$ *str*$^s$ in one-to-one proportion under conditions of prolonged mating (in order to allow transfer of the factor responsible for MS2 sensitivity to many recipient bacteria). A sample of the mating mixture was diluted and plated on minimal medium supplemented with the appropriate amino acids so that only C600 cells could grow. Fifty colonies were picked, their cells were purified by a second passage on the same selective medium, and tested with phage MS2. All 50 strains had acquired sensitivity to MS2 phage, indicating that the factor was efficiently transferred.

One of these strains was tested further. It had acquired neither ability to produce colicins V or I nor immunity to either colicin, though it was sensitive to the male specific phage. We shall hereafter refer to the factor present in these strains as $F_x$.

Strain C600($F_x$) was in turn employed as donor in mating experiments with the $F^-$ strains AB1157/V,I and PA309/V,I as recipients and different types of recombinants were selected (Table 2). As seen in Table 2, a frequency of recombination of about $10^{-6}$ was observed in crosses involving strain C600($F_x$) as donor, of the same order as in a cross with a C600(colV colI) donor. No recombinants were found in control crosses C600 × AB1157/V,I or C600 × PA309/V,I.

TABLE 2

*Comparison of the frequency of recombination in matings involving strains C600($F_x$) and C600(colV colI) as donors*

| Crosses | Frequency of recombinants per donor cell | | | |
|---|---|---|---|---|
| | *arg*$^+$*str*$^r$ | *his*$^+$*str*$^r$ | *pro*$^+$*str*$^r$ | *try*$^+$*str*$^r$ |
| C600 *str*$^s$ × AB1157/V,I | 0 | 0 | 0 | . . |
| C600 *str*$^s$($F_x$) × AB1157/V,I | $8.5 \times 10^{-6}$ | $2.3 \times 10^{-6}$ | $1.6 \times 10^{-5}$ | . . |
| C600 *str*$^s$ × PA309/V,I | 0 | 0 | . . | 0 |
| C600 *str*$^s$($F_x$) × PA309/V,I | $2.6 \times 10^{-6}$ | $1.3 \times 10^{-6}$ | . . | $1.3 \times 10^{-6}$ |
| C600 *str*$^s$(colV colI) × PA309/V,I | $3 \times 10^{-6}$ | $2 \times 10^{-6}$ | . . | $8 \times 10^{-6}$ |

Strains were mixed in the proportion of one donor to ten or 20 recipients. The frequency of recombinants was measured at 120 min after the onset of mating.

Strains carrying the $F_x$ factor showed, by spot test, the same restriction against P1 as strains carrying colV colI.

*Isolation and study of strains resistant to MS2:* Mutants resistant to phage MS2 were isolated in an attempt to select strains that carried the colV colI factors without the fertility determinant. Twenty such derivatives from strain AB1122 (colV colI) were studied. Nine of them, which were resistant also to phages f1 and $Q\beta$, and still synthesized both colicins, were employed as donors in mating. Three of these strains still transferred the colV colI factor, though $10^3$ to $10^4$ times less efficiently than the original strain AB1122(colV colI) $MS2^s$; two other strains transferred it even less efficiently; the remaining four strains did not mediate the transfer of colV colI at any detectable rate.

In six of these strains the loss or reduction of fertility was shown to be due to a mutation in the factor and not to a chromosomal mutation, by the following observations: (a) When the colV colI factor was transferred from those cells showing residual fertility to other $F^-$ cells the resulting colicinogenic bacteria did not acquire sensitivity to MS2 phage. (b) On elimination of the colV colI by acridine orange treatment and subsequent reinfection with the original colV colI plasmid, the cells regained sensitivity to phage MS2. The mutated factors present in these strains was designated colV colI $MS2^r$.

*Exclusion between $F_x$ and colV colI $MS2^r$:* Strain C600($F_x$) was mated with several of the strains AB1122(colV colI $MS2^r$) under conditions of prolonged mating. Samples of the mating mixture were diluted and plated on a selective medium where only strain AB1122 could grow. Colonies were picked, transferred to the same selective medium, and tested for acquisition of sensitivity to phage MS2. Eighty-eight colonies were analyzed from a mating experiment involving one of the AB1122(colV colI $MS2^r$) strains, and 55 colonies from mating involving five other such strains. Among these 143 colonies, 139 had not acquired sensitivity to the MS2 phage and produced both colicins; the remaining four, which had acquired it, had simultaneously lost the ability to synthesize colicins V and I. In a control cross C600($F_x$) × AB1122, performed under the same conditions, all of 68 colonies of recipient type tested had received the $F_x$ factor. These observations indicate the existence of interference between the $F_x$ factor and the mutated plasmid colV colI $MS2^r$.

*Transfer of the colV colI factor to a $colI_a^+$ recipient strain:* After mutagenesis of AB1122(colV colI) by nitrosoguanidine, some colonies were isolated which synthesized only colicin V but were still immune to both colicins V and I and sensitive to phage MS2. These strains, which may carry a defective colI factor, are called AB1122 (colV $colI^d$). In mating, the colV $colI^d$ factor is transferred as frequently as the normal colV colI.

Crosses were performed between a strain carrying colV $colI^d$ or the normal colV colI and various strains $F^-$ or $F^-(colI_a)$ as recipients, in order to see if any interference between the colV colI factor and $colI_a$ occurred. As seen in Table 3, comparable frequencies of transfer were observed for colV colI or colV $colI^d$ factors in these matings, irrespective of whether or not the $F^-$ strain carried $colI_a$.

Recombinants $colV^+$ str-r from these crosses were isolated and ten colonies

TABLE 3

*Transfer of* colV colI *or* colV colI$^d$ *plasmids to recipients carrying* colI$_a$

| Crosses | Frequency of colV$^+$ *str$^r$* cells |
|---|---|
| AB1122(colV colI) × PA309/V,I | 950 |
| AB1122(colV colI) × AB1157/V,I | 1000 |
| AB1122(colV colI) × PA309/V,I(colI$_a$) | 1050 |
| AB1122(colV colI$^d$) × PA309/V,I | 500 |
| AB1122(colV colI$^d$) × AB1157/V,I | 350 |
| AB1122(colV colI$^d$) × PA309/V,I(colI$_a$) | 400 |

Matings were performed in the proportion of one donor to ten recipient cells. The frequency of colV$^+$ *str$^r$* cells, measured 120 min after the onset of mating, is expressed as percent ratio of the number of these cells to the initial number of donor bacteria. (ColI$_a$ is the colI factor derived from *E. coli* CA53.)

from each isolation plate were tested for production of colicins V and I. No segregation of the colV$^+$ property was observed among 30 isolates from cross AB1122 (colV colI) × PA309/V,I(colI$_a$). The high frequency of transfer and the absence of segregation indicate that, as far as the colV colI factor is concerned, no exclusion to its entry and persistence is exerted by a colI$_a^+$ recipient strain. In crosses involving strains AB1122(colV colI$^d$) and PA309 (colI$_a$), out of 33 colV$^+$ *str$^r$* isolates analyzed, three segregated some colI$^+$ bacteria which did not produce colicin V, indicating a loss of the colV colI$^d$ factor. All the others synthesized both colicins V and I and appeared to carry both the colV colI$^d$ and the colI$_a$ plasmids.

One of the strains, AB1157(colV colI$^d$)(colI$_a$), was used as donor in mating with strain C600 *str$^s$*. Samples were taken at 2 hours and after prolonged mating. Out of 40 colonies of C600 type tested from the 2-hour sample, 21 produced only colicin V. All 120 colonies tested after prolonged mating also produced colicin V and not colicin I. This result shows that the colV colI$^d$ plasmid is transferred with high efficiency while the colI$_a$ factor is transferred with much lower frequency or not at all. It also indicates that the persistence of these two plasmids in one cell is not due to the occurrence of a recombinational event.

## DISCUSSION

In the $P^{32}$ decay experiment the colV colI plasmid gave rise to an F-like factor, $F_x$, which produces neither colicin V nor I and does not confer immunity to either of them. It is likely that $P^{32}$ decay resulted in a physical separation of $F_x$ from colV colI, although the possibility of a mutation suppressing the functions of both colV and colI cannot be excluded.

Like the original colV colI, the $F_x$ factor is transferred with high efficiency to F$^-$ cells and imparts to them a low level of chromosomal fertility. These findings support the conclusion that the fertility properties determined by colV colI are not due to the colicinogenic factors themselves, but to an F factor closely attached to them. It seems, therefore, appropriate to designate the colV colI plasmid as $F_x$ colV colI. On the basis of somewhat different lines of evidence, KAHN and HELINSKI (1965) have reached similar conclusions with regard to a plasmid called $F_v$ colV, which might be related to $F_x$ colV colI.

Our results, together with the observation that the $F_x$ factor is itself capable of autonomous multiplication and transfer, suggest that the replication of the $F_x$ colV colI plasmid is under the control of the $F_x$ replicator. In fact, $F_x$ competes with the intact $F_x$ colV colI plasmid in the same way as do F-*lac* or F-*gal* (NAGEL DE ZWAIG and ANTÓN 1964), whereas no interference is observed between $F_x$ colV colI and the $colI_a$ factor. STOCKER (1965) did find a strong interference between the $colI_a$ and $colI_b$ factors, which presumably replicate under similar control mechanisms.

Exclusion phenomena have been observed to occur also between F-*lac* and F-*gal* (DE HAAN and STOUTHAMER 1962; ECHOLS 1963), among R factors (WATANABE *et al.* 1964), and among plasmids controlling the synthesis of penicillinase in Staphylococcus (NOVICK and RICHMOND 1965).

Two different models may be considered to explain exclusion between plasmids: a repressor model, exemplified by the block of replication of a temperate phage superinfecting an immune bacterium, and a competitive model, in which similar replicating units or "replicons" may compete for a number of replicative bacterial sites, possibly on the bacterial membrane (JACOB, BRENNER, and CUZIN 1963).

*Colicinogenic* factors, like temperate phages, can apparently multiply in unrestricted fashion after treatment with inducing agents (AMATI 1964; DEWITT and HELINSKI 1965) and sometimes also after entering a new host cell, as shown for colI by the occurrence of high-frequency colicinogenic transfer (STOCKER, SMITH and OZEKI 1963; CLOWES 1964). Yet, in stably colicinogenic bacteria the *col* factors presumably replicate in a restricted manner, although they are not integrated in the bacterial chromosome (NAGEL DE ZWAIG *et al.* 1962). Thus, an independently replicating plasmid can exert a specific repression over its own replicator system. This may be the case also for phage P1, which appears to have no chromosomal location, yet establishes a specific immunity mechanism (BOICE and LURIA 1963).

For the F factors, even though the rapid spread of fertility in a newly infected population suggested a phase of unrestricted replication, no evidence for it was found in a study of F-*lac* transfer (REVEL 1965).

In the case of the composite plasmid $F_x$colV colI, the absence of interference with $colI_a$ and the presence of interference with F factors suggest that this plasmid replicates under the control of its F component. The fact that colicin I production can be induced by ultraviolet irradiation of strains carrying $F_x$colV colI may be interpreted as indirect evidence of a release of replication control, either of the whole plasmid or of its colI component, since it has been reported that irradiation causes extensive multiplication of colI in *E. coli* cells (AMATI 1964).

The occurrence of mutant plasmids colV colI $MS2^r$, which determine synthesis of the colicins without sensitivity to male specific phages, is a clear indication that the colicinogenic properties can be functionally separated from the fertility properties. In these strains the $F_x$ factor still persists, though in a defective state, as shown by its ability to interfere with an entering $F_x$.

The fact that the fertility effect can be separated from the colicinogenic determinants present in the same plasmid, as shown by the present observations with

$F_x$colV colI and also for colV and colB by FREDERICQ (1963b), suggests a similarity between these plasmids, the F′ factors, and also the R factors, whose determinants of drug resistance can be separated from the fertility determinants (SUGINO and HIROTO 1962). F′ plasmids are known to originate by recombinational events between an F factor and the chromosome (JACOB and ADELBERG 1959; ADELBERG and BURNS 1960) or between an F factor and an R plasmid (HARADA *et al.* 1964). The $F_x$colV colI plasmid may have been formed in a similar way by recombinational events between an F factor and the colicinogenic determinants. Transfer of chromosomal genes is, in fact, promoted by the colI factor (OZEKI *et al.* 1962; CLOWES 1964); hence, some region of homology suitable for recombination between colI and the F factor may well exist. Recombinational events may also occur between an F col plasmid and an F or F′ factor. FREDERICQ (1963a) has isolated an Hfr strain carrying the colB factor at the distal end of the chromosome as a result of a close association with the integrated F factor. KAHN and HELINSKI (1965) have observed interactions between $F_v$colV and the F region of an Hfr chromosome, which probably originate by recombinational events. The existence of homologous regions in these various plasmids could probably be tested by hybridization tests with isolated DNA fractions.

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## SUMMARY

The fertility conferred on a cell by a colV colI plasmid is due to a factor distinct from the colicinogenic determinants. This fertility determinant can be physically or functionally separated from the colicinogenic property as shown by: (a) Isolation from a $P^{32}$ decay experiment of an F factor ($F_x$) which is transferred with the same efficiency and confers the same degree of chromosomal fertility as the whole colV colI plasmid but does not determine production of, or immunity to, colicins V and I; and (b) isolation of mutants carrying the colV colI factor but resistant to male specific phages. These mutants still harbor a defective F since exclusion of an entering $F_x$ factor is still observed. These results, together with the observed interference between $F_x$colV colI and the F factor and the lack of interference with the $colI_a$ factor, suggest that the $F_x$colV colI plasmid replicates in the bacterial cell under the control of the F replicator.

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