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ColB2-K77, a Fertility-Repressed F-Like Sex Factor

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The colicinogenic B factor, transferred from *Escherichia coli* strain K77 (and termed ColB2-K77 or ColB2) to an *E. coli* K12 F^- strain, is capable of promoting its own transfer to other K12 F^- strains at a low rate (from LFC cultures) which can be increased under special conditions (HFC cultures). LFC cultures of K12 $(ColB2)^+$ F^- strains show a low level of adsorption of F-specific phage particles which also increases under HFC conditions. The ColB2 factor is thus inferred to be an F-like sex factor which is repressed in its fertility. This repression is concluded to be due to a cytoplasmic repressor since, when ColB2 is present in cells containing an F factor (either autonomous or integrated), F fertility is also repressed as shown by the inability of such $(ColB2)^+F^+$ [or $(ColB2)^+$Hfr] strains to plaque F-specific phages, and by a reduction in the level of chromosomal transfer from such strains, compared to the corresponding F^+ (or Hfr) control strains. Mutants of the ColB2 factor in which fertility is no longer repressed (fertility derepressed or Fdr mutants) have been isolated. The ColB2Fdr mutant strains do not appear to be able to mobilize chromosomal transfer, although they have acquired F-specific phage sensitivity demonstrable by plaque formation and they transfer their colicin factor at high frequency and are well piliated. The Fdr mutation is presumed to result in the inability to synthesize the cytoplasmic fertility repressor since the ColB2Fdr factor does not repress the fertility of an F factor when present in the same host strain. A fertility-repressed drug resistance factor of the R(f) type is not stable in the presence of a ColB2 factor in the same cell and is eliminated in about 10% of the cells per generation. In contrast, another factor characteristic of the R(i) type is fully compatible with ColB2. Under conditions artificially stabilizing $(ColB2Fdr)^+$ $(Rf)^+$ strains, the enhanced fertility of ColB2Fdr is not repressed by the presence of the R factor, nor does the presence of R(f) in the intermediate strain of an HFC (for ColB2) system inhibit the normal increase in ColB2 transmissibility. It is concluded that the repressors of R(f) and ColB2, although both active on F fertility, are different; this may indicate that at least two independently repressible cistrons are involved in the expression of fertility characteristics.

Early concepts of bacterial sex factors were based largely on studies of the classical factor F (8). However, studies of other sex factors, such as colicin (Col) factors and infectious multiple drug resistance (R) factors, have shown that the ability of F to express its fertility in virtually all of its host cells is the exception rather than the rule. Most other sex factors appear to be repressed in that they do not normally express their fertility in the majority of host cells (13). From many of these self-repressed sex factors, mutants which are derepressed in their fertility have been obtained (4, 12).

This paper analyzes some characteristics of the colicinogenic factor isolated from *E. coli* K77 (termed ColB2-K77, in this paper abbreviated as ColB2) which appears to be a sex factor of the self-repressed fertility type. The interaction of this factor and its fertility-derepressed (Fdr) mutants when present in the same cell with F-*lac*, integrated F, or an F-like or I-like R factor has been studied. Preliminary reports of these studies have already appeared (2; Clowes, Hausmann, Nisioka, and Mitani, J. Gen. Microbiol. **55**: iv, 1969; Hausmann and Clowes, Bacteriol. Proc., p. 54, 1968), and since then other workers have confirmed some of these initial observations (6).

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MATERIALS AND METHODS

Bacterial strains. The characteristics of the strains used are summarized in Table 1. The source of the colicin B factor was the *E. coli* strain K77 of Frédéricq (5). The R factors used comprised 222/R4, an F-like R factor carrying resistance to streptomycin, sulfonamide, chloromycetin, and tetracycline, and R15, an I-like factor carrying resistance to streptomycin and sulfonamide (16). Both R factors are normally repressed in their fertility.

Media. Nutrient broth, nutrient agar, minimal agar, supplemented minimal agar, soft agar, and eosin methylene blue (EMB) media were prepared as referred to elsewhere (14). Tryptone broth contained, per liter, 10.0 g of tryptone (Difco), 5.0 g of NaCl, 1 mmole of $MgSO_4$ (pH 7.3, adjusted with NaOH).

General techniques. The following techniques were described by Monk and Clowes (14): assays for colicinogeny, titration of free colicin, isolation of colicinogenic strains, kinetic measurements of transfer of colicinogeny (at low and high frequencies), testing for F-phenotype (fertility characteristics), F^+ and Hfr-mediated recombination. Other techniques were as follows.

Adsorption test of male-specific phage sensitivity. A 0.1-ml amount (10^{11} plaque-forming units/ml) of a suspension of phage $\mu 2$ (3) was added to 1 ml of the exponential (2×10^8 cells/ml) bacterial culture to be tested, previously grown in L broth supplemented with calcium (10) and held at 37 C. After 5 to 10 min at 37 C, unadsorbed phage were removed by a 1:10 dilution into anti-$\mu 2$ serum ($K \sim 6$) and held at 37 C for a further 5 min. Samples (0.1 ml) were diluted in L broth and incubated at 42 C. Samples before and after incubation were overlayered on L agar in soft agar together with ca. 10^9 cells of RC732, a μ-s indicator strain, to assess μ particles.

Spot test of male-specific phage sensitivity. One drop of an exponentially growing culture to be tested was spread on a sector (½ to ⅙) of a predried nutrient agar plate. When the spot had dried, a droplet of a suspension of phage $\mu 2$ (titer 10^{11} particles per ml) was placed on the bacterial film with a Pasteur pipette. After 8 to 12 hr of incubation at 42 C, the areas of bacterial growth were examined for lysis.

Isolation of ColB2 Fdr mutants. The selection technique for Fdr mutants of ColB2 was based on the fact that ColB2 can coexist stably in the same cell with an F factor but inhibits its fertility. A derepressed ColB2 factor (lacking fertility repressor, i.e., of the i^- type), however, would not be expected to show such inhibition and could be identified by screening for cells with high fertility in a mutagen-treated culture of a strain harboring ColB2 and F-*lac*. Strain RC24 (*thr leu thi lac*$^-$ *str-r*) was thus infected with ColB2 and F-*lac*$^+$ by two successive matings with RC416 and RC770, respectively. An overnight culture in tryptone broth of this strain was diluted to a concentration of 10^4 cells per ml into fresh tryptone medium containing 1.5 mg of 2-aminopurine per ml and aerated at 37 C until a concentration of about 5×10^8 cells/ml was reached. The culture was diluted fivefold into nutrient broth, further incubated for 2 hr at 37 C, again diluted, and plated on nutrient agar master plates (about 200 cells/plate). When microcolonies (containing about 10^4 cells) had developed, two replicas were made on lactose-minimal medium plates supplemented with proline and methionine, and on which 0.1 ml of an overnight culture of strain RC776 (*pro met lac*$^-$) had been spread. After 48 hr of incubation, the master colonies which had given rise to replica colonies [of RC776 infected with F'-*lac*$^+$ from RC24 (ColB2)$^+$(F-*lac*$^+$)$^+$] on duplicate replica-plates were picked and tested for the presence of colicinogeny (B) and $\mu 2$ sensitivity, indicating a derepressed ColB2 factor. F'*lac* was then eliminated from these tested colonies by acridine orange treatment (7).

RESULTS

Transfer of the ColB2 factor. When logarithmic cultures of the wild-type *E. coli* K77 strain (RC416) and a K12 F^- strain (RC578) were mixed in equal volumes and incubated at 37 C, transfer of ColB2 to the K12 recipient reached about 5% after 2 hr. A similar frequency of transmission [termed low-frequency colicinogeny transfer (LFC)] was also observed from these K12 ColB2-infected strains to further K12 F^- recipients (Fig. 1A). When a high-frequency colicinogeny (HFC) transfer system was set up involving three K12 strains, transfer increased reaching about 15% after 1 hr of contact (Fig. 1B).

F-specific phage adsorption. The ability of stably infected (ColB2)$^+$ K12 strains (LFC cultures) to adsorb the F-specific phage $\mu 2$ was compared with an Hfr strain and also with an HFC culture of the same ColB2 factor. In con-

TABLE 1. *Escherichia coli* strains used

Type	Collection no.	Genotype	Origin or synonym
E. coli K12	RC15	*thr leu thi lac*$^-$ *azi-r str-r* F^-	W1
	RC24	*thr leu thi lac*$^-$ *str-r* F^-	W945
	RC578	*met azi-r* F^-	58-161
	RC588	*met azi-r* (222/R4)$^+$	RC578 × *E. coli* CSH2 (222/R4)$^+$
	RC589	*met azi-r* (R15)$^+$	RC578 × *E. coli* CSH2 (R15)$^+$
	RC712	*pro his trp lac*$^-$ *str-r* F^-	J62
	RC732	Prototroph HfrH	A'Hfr (recombinant of Hfr HAYES)
	RC770	*met* (F'-*lac*)$^+$	W1655 infected with F-*lac*$^+$ from HfrP4X
	RC776	*pro met lac*$^-$ *str-r* F^-	Recombinant W1 × Hfr Cavalli
Other *E. coli*	RC416	Prototroph (ColB)$^+$	K77 (6)

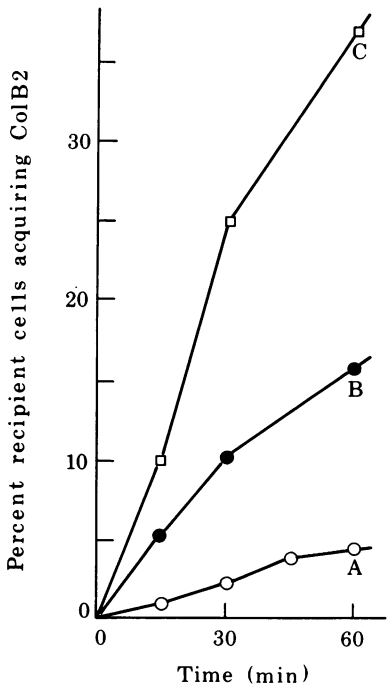

FIG. 1. *Kinetics of ColB2 transfer. Curve A, Transfer of ColB2 (wild) under LFC conditions (○); donor RC578(ColB2)⁺, recipient RC15. Curve B, Transfer of ColB2 (wild) under HFC conditions (●); donor RC578(ColB2)⁺, intermediate RC578, recipient RC15. Curve C, Transfer of ColB2Fdr (□); donor RC578(ColB2Fdr)⁺, recipient RC15. In crosses A and C, 1-ml samples of donor and recipient, each in the exponential phase of growth at 2 × 10⁸ cells/ml were mixed and incubated without shaking at 37 C. At the times shown, 0.1-ml samples were removed, diluted, and spread on nutrient agar containing streptomycin by the double-overlay method (14). After overnight incubation, the plates were overlayered with soft agar, containing the ColB-s str-r indicator strain (RC712). After further incubation, the proportion of colonies showing inhibition zones of the indicator was determined. In the cross shown in curve B, 5 ml of nutrient broth was inoculated with 5 × 10⁴ donor cells and 10⁶ intermediate cells and incubated overnight. A 1:20 dilution was then made to fresh nutrient broth and reincubated to exponential phase at approximately 2 × 10⁸ cells/ml. A 1-ml amount of this HFC mixture was then added to 1 ml of 2 × 10⁸ recipient cells/ml, also in exponential phase, and the above procedure was followed.*

trast to an Hfr strain, where 53% of the cells adsorbed phage yielding a progeny of about 14,000 phages per infected cell, approximately 10% of the cells in an HFC culture adsorbed the phage, producing a progeny of about 5,000 particles per infected cell. In the LFC stage, the adsorption was less than 1%, and no phage production was detected. Sensitivity was more generally investigated by spot test. Indicator strains harboring a ColB2 factor showed no $\mu 2$ lysis when used either in the LFC or HFC state or when present in the same host as an F-*lac*⁺ factor. When ColB2 was present in an Hfr strain, lysis was barely visible. In contrast, (ColB2Fdr)⁺ strains showed clear areas of lysis similar to those shown by an Hfr or F-*lac*⁺ indicator strain, as did Hfr and F-*lac*⁺ strains to which this factor had been transferred. Moreover, similar lysis was observed of an indicator strain harboring both a ColB2Fdr factor and the fertility-repressed 222/R4 factor.

Interaction of ColB2 with other factors: F'lac. The kinetics of transfer of an F'*lac* factor to a (ColB2)⁺F⁻ recipient were similar to those found using the corresponding F⁻ strain as recipient. The stability of the F'*lac* and ColB2 factors in the common host was investigated by comparing an isolate of strain RC24 (Lac⁻) infected by F'*lac*⁺, with 20 independent isolates of RC24(ColB2)⁺ infected with F'*lac*⁺, as follows. A single colony from each strain was suspended in buffer and diluted to about 10^4 cells per ml; samples were spread on EMB-lactose or nutrient agar plates. In all cases, all of the resulting colonies were both uniformly nonsectored and stably lactose positive, and those from the ColB2 recipients were also stably colicinogenic, producing normal sized inhibition zones. Similar results were obtained after several serial subcultures of an RC24(ColB2)⁺(F'*lac*⁺)⁺ strain. The (ColB2)⁺(F'*lac*⁺)⁺ strains, however, no longer showed sensitivity to the F-specific phage $\mu 2$ in a spot test, and transmission of F'*lac* to F⁻ recipient strains was 20-fold lower than from the corresponding RC24(F'*lac*)⁺Col⁻ donor, indicating that the fertility of F'*lac* was reduced by the presence of ColB2 in the same cell.

Hfr. In a similar way, transfer of ColB2 to the Hfr strain, HfrH, as a recipient was as efficient as to an F⁻ recipient. Chromosomal transfer from the Hfr(ColB2)⁺ strain so produced was reduced about 10-fold compared to the transfer from the control HfrH to the same F⁻ recipient (Table 2).

R factors. Strains containing the R factors R15 or 222/R4 were each as efficient a recipient for ColB2 as was the corresponding R⁻ strain (5% transfer in 2 hr). The (R15)⁺(ColB2)⁺ strains so produced were stable for both factors when tested similarly to (F'*lac*)⁺(ColB2)⁺ as described previously. (In this case, the presence of R15 was assessed by scoring plasmid-controlled streptomycin resistance on nutrient agar containing 100 μg of streptomycin per ml.) However, when 50 independent isolates of a (222/R4)⁺(ColB2)⁺ strain were similarly tested,

TABLE 2. *Transfer of chromosomal genes from HfrH and its ColB2⁺ derivatives*[a]

Donor	Recombinants per donor cell		
	pro⁺	*trp⁺*	*his⁺*
HfrH	3.5×10^{-3}	6.0×10^{-4}	1.0×10^{-4}
HfrH(ColB2)⁺	3.5×10^{-4}	6.6×10^{-5}	7.2×10^{-6}
HfrH(ColB2Fdr)⁺	5.1×10^{-3}	1.6×10^{-3}	3.4×10^{-4}

[a] Crosses of donor HfrH strain RC732 or its ColB2⁺ or ColB2Fdr⁺ derivatives with recipient strain RC712.

either for tetracycline or chloromycetin resistance (controlled by R4), many daughter colonies had lost their resistance to these drugs and to streptomycin and sulfonamide, the resistances of which are also controlled by R4, although all colonies were found to be colicinogenic. The fate of the R factor was followed more closely in one clone and showed that the factor is not immediately eliminated, but is lost through successive generations with an approximate frequency of 10% per generation (Fig. 2).

Isolation and characterization of fertility-derepressed ColB2 mutants. The ability of F'*lac* to coexist stably in the same cell with ColB2 under which circumstances F fertility is reduced, served as a basis for the isolation of fertility-derepressed ColB2 (i.e., ColB2Fdr) factors, as detailed above. Seven Fdr mutants were isolated, and one mutant was studied in detail. The transmission of this factor from a K12 donor strain to an F⁻ recipient was more efficient than from an HFC culture carrying the wild (fertility-repressed) factor (Fig. 1C), approximately 40% of the F⁻ cells inherited the derepressed ColB2 factor in 60 min. The Fdr mutant was also susceptible to F-specific phage, as judged by the ability of $\mu 2$ to produce plaques or give positive results in spot tests when strains carrying the ColB2Fdr factor was used as hosts. Electron micrographs of ColB2Fdr cells showed several pili per cell, whereas the corresponding strain carrying the wild-type (repressed) ColB2 factor showed no such piliation (Fig. 3). Attempts to demonstrate chromosomal transfer from donor strains carrying ColB2Fdr were unsuccessful; however, the frequency of recombinant formation was measured only as less than 10^{-7} per donor cell. When ColB2Fdr was present in an Hfr strain, chromosomal transfer was slightly higher than from the original Hfr strain (Table 2), indicating the absence of fertility repression by ColB2Fdr.

When (ColB2Fdr)⁺ strains were infected with 222/R4, there appeared to be no loss of fertility, as seen by the retention of F-phage sensitivity. Maintenance of the R factor in the culture was assured by the presence of 50 μg of chloramphenicol per ml in the medium during tests; checking for the R factor at the end of the experiment confirmed the efficacy of this procedure. A further test of the lack of susceptibility of ColB2 to the 222/R4 repressor was carried out as follows. An HFC culture with the wild-type ColB2 factor was derived by using a strain carrying the 222/R4 factor as an intermediate. As can be seen in Fig. 4, transfer of ColB2 from this strain to the recipient was as efficient as when a control intermediate strain without the 222/R4 factor was used.

DISCUSSION

Fertility repression of F, R(f), and ColB2. The low level of $\mu 2$ phage adsorption and self-transfer of ColB2, both of which are increased under HFC conditions (Fig. 1), qualify ColB2 as an F-

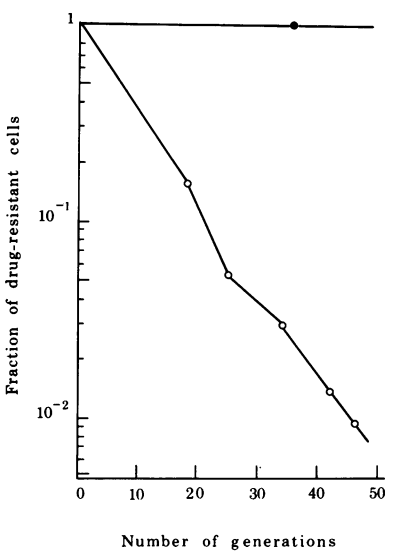

FIG. 2. *Elimination of drug resistance in a (ColB2)⁺ (222/R4)⁺ strain.* Single colonies of the doubly infected *RC578(ColB2)⁺(222/R4)⁺ strain* (○) *and of the parental RC578(222/R4)⁺Col⁻ strain* (●) *were separately suspended in 1 ml of prewarmed nutrient broth, diluted to about 10^{-3} to give approximately 10^5 cells/ml, and incubated at 37 C to about 10^8 cells/ml. The culture was again similarly diluted and reincubated, and this process was repeated until about 50 generations of growth had occurred. (Calculated by $N_t = N_0 2^n$, where N_t is final number of cells, N_0 is the original inoculum, and n is the total number of generations.) At various times during growth, samples were diluted and plated, either on media supplemented with tetracyclin (50 μg/ml) or chloromycetin (50 μg/ml) or on nutrient agar. After overnight incubation, the number of colonies on the drug plates were counted, and those on drug-free medium overlayered with a ColB-s indicator strain to identify colicinogenic colonies.*

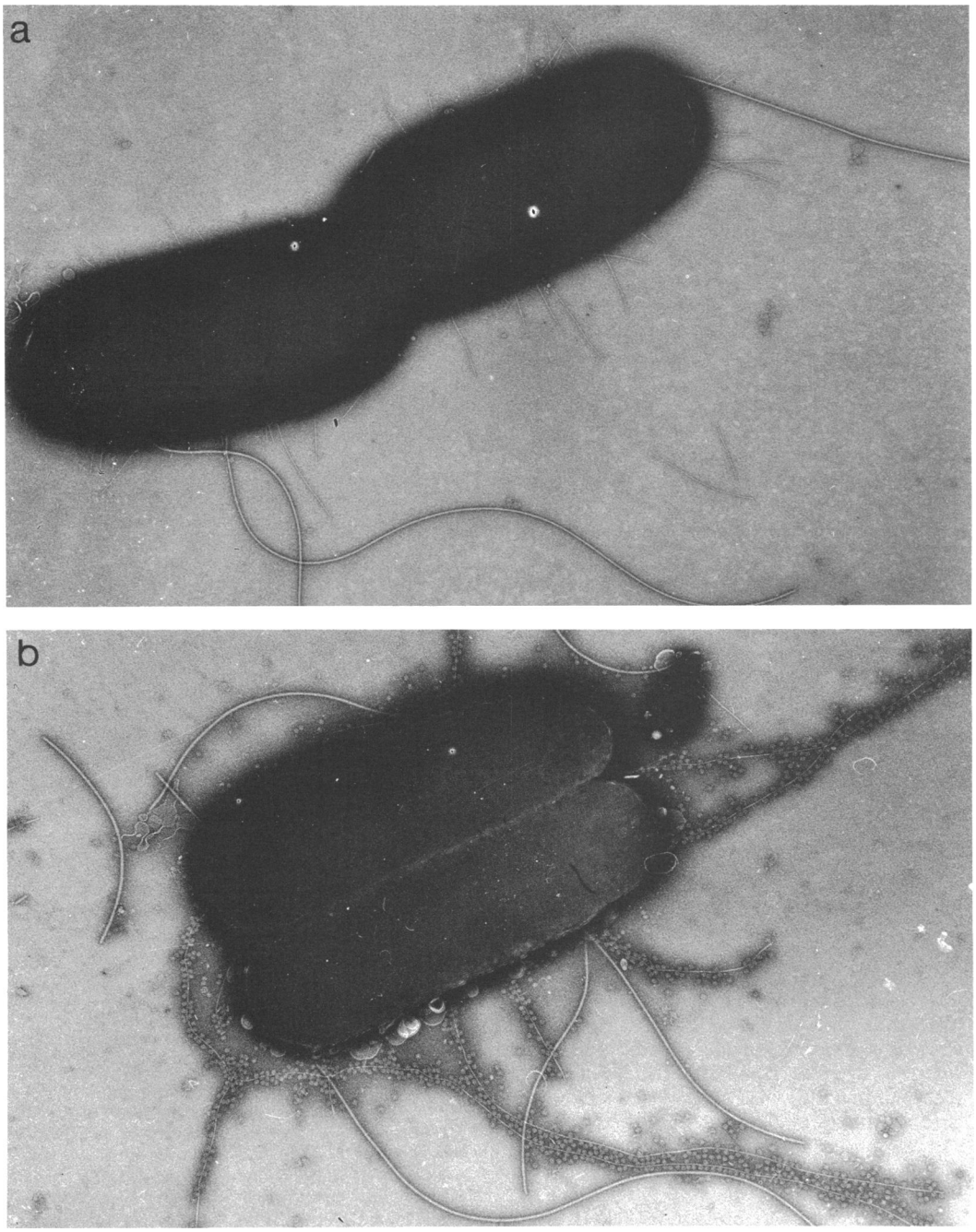

FIG. 3. *Electron micrograph of* $(ColB2)^+$ *and* $(ColB2Fdr)^+$ *cells. To exponentially growing cultures of* $RC578(ColB2)^+$ *and* $RC578(ColB2Fdr)^+$, *μ2 phage at a multiplicity of about 500 plaque-forming units/cell were added. Samples were negatively stained with phosphotungstic acid. a,* $(ColB2)^+$, × *24,000; b* $(ColB2Fdr)^+$, × *33,000. (Photographs were kindly supplied by Dimitrij Lang.)*

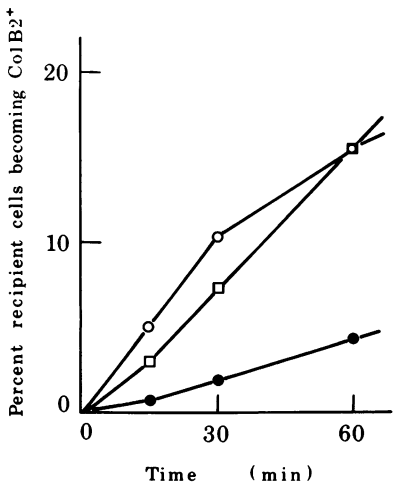

FIG. 4. *High-frequency ColB2 transfer in the presence of the fertility repressor of 222/R4. HFC mixtures using the donor strain RC578(ColB2)$^+$ with each of the three intermediate strains RC578* (○), *RC578(ColB2)$^+$* (●), *or RC578(222/R4)$^+$* (□) *were prepared; ColB2 transfer to the same recipient strain, RC15, from each of the three HFC mixtures was investigated, as detailed in Fig. 1. In the HFC mixture using the (222/R4)$^+$ intermediate strain, the nutrient broth medium was supplemented with 50 μg of tetracycline during the overnight incubation of donor and intermediate strains.*

like sex factor with self-repressed fertility. ("F-like" is used in this context to imply a sex factor producing pili to which F-specific phages can adsorb.) Repression of ColB2 fertility appears to be due to a cytoplasmic substance since it extends to an F factor present in the same cell. The isolation of Fdr mutants of ColB2 showing enhanced self transfer and F-phage sensitivity may be due either to mutation in the structural repressor gene (*i*-type mutant) or in the putative (operator) locus on which this gene product may act (o^c-type mutant). The former possibility is supported by the lack of repression of F fertility by ColB2Fdr. However, when an R(f) factor is introduced into the same strain as ColB2Fdr, R(f) does not repress ColB2 fertility as it does that of F (16). The fertility-operator locus of ColB2Fdr does, therefore, not appear to be susceptible to the R(f) fertility repressor. This could also be concluded for the wild ColB2 factor by the fact that the presence of R(f) in an intermediate strain of an HFC mixture for the wild ColB2 factor does not prevent its epidemic spread, which it might be expected to do were it able to repress ColB2 fertility.

One possible explanation of these results would be that the products of several genes, each with its own operator locus, are necessary for fertility. If R(f) and F have one operon in common which is sensitive to the R(f) repressor and if ColB2 and F have a different operon in common, sensitive to the ColB2 repressor, this would accommodate the experimental data. Kétyi and Ørskov have recently shown (9) antigenic similarities in F, R(f), and ColB2. They propose that these factors produce at least one common antigen in addition to other antigens which are specific for each factor. Table 3 shows a scheme incorporating Kétyi and Orskov's findings and the present observations. Since repression by either R(f) or ColB2 leads to repression of pilus formation, the antigenic components could be supposed to be structural components of the pilus.

TABLE 3. *Possible fertility control interactions between R(f), F, and ColB2*

Sex factor	Sex-factor antigens[a]	Repressor for genes	Repressor-sensitive genes
F	abf		ab
R(f)	acf	a	a
ColB2	bdf	b	b

[a] f, Common antigen which may possibly represent male-specific phage attachment site.

Incompatibility of ColB2 and R(f). The inability of R(f) to be maintained stably in a cell harboring a ColB2 factor is similar to the situation previously reported between ColV3 and F and between F and ColV2 (11). This phenomenon has been described by Novick (15) as "plasmid incompatibility" since there is clear evidence in all cases that "entry exclusion" does not in fact occur. Furthermore, this phenomenon can be clearly distinguished from "restriction" where the deoxyribonucleic acid of the transferred factor is physically degraded (1, 17). One possible explanation would invoke cytoplasmic repression of plasmid replication. Here again, a similar explanation used to explain fertility control would be compatible with the data. That is, if several gene products were necessary for replication, a gene common to R(f) and ColB2 (or to F and ColV2) would be the target for repression by ColB2 (or ColV2), whereas R(f) (or F) would be self-regulated by repression of a replication gene not held in common with ColB2 (or ColV2).

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