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Genetic and Molecular Characteristics of Vir Plasmids of Bovine Septicemic *Escherichia coli*

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Three wild strains of bovine septicemic *Escherichia coli* were selected on the basis of their production of a toxin lethal for mice and chickens and their characteristic surface antigen. The transfer of these virulence (Vir) properties from two of the three to recipient *E. coli* was detected after mating. One Vir plasmid (pJL1) was derepressed for transfer and associated with mobilization of chromosomal markers. The other, pJL2, was repressed. Both plasmids were tagged with transposon Tn*5* (kanamycin resistance), and transfer parameters of the tagged plasmids were studied. The Tn*5* insertion in pJL2 usually increased transfer efficiency 100-fold. Plasmid pJL1 was classified as a member of the FIV incompatibility group. A pJL1::Tn*5* derivative plasmid was incompatible with ColV1. Plasmid pJL2 behaved as an fi$^+$ plasmid. Both plasmids pJL1 and pJL2 had a molecular weight of 92×10^6 and were present at about four copies per chromosome; their deoxyribonucleic acid (DNA) structures were not identical on the basis of restriction enzyme analysis. DNA-DNA hybridization revealed a polynucleotide sequence homology of at least 58% between the two plasmids. No plasmids could be detected in one wild or certain laboratory-derived Vir$^+$ *E. coli* strains.

On the basis of genetic evidence (26, 27, 30), two virulence plasmids were detected by Smith and Huggins in invasive *Escherichia coli* of animal and human origins. One of the two, plasmid ColV, has been shown to endow bacteria with increased virulence (27, 30) by virtue of greater ability to resist the defense mechanisms of the host. The other virulence plasmid discovered by Smith (26, 27) in septicemic *E. coli* strains is the Vir plasmid. The Vir phenotype is characterized by the synthesis of a toxin and a surface antigen. Broth culture supernatants or sterile lysates of Vir$^+$ *E. coli* are lethal for chickens and produce characteristic macroscopic and histological lesions very similar to those caused by the chick lethal toxin of septicemic *E. coli* (34, 35). It is likely that both toxins are the same or very closely related.

One of the difficulties in studying virulence plasmids is that there is usually no suitable selection mechanism for detection of their transfer. The first objective of this study was to determine the transfer characteristics of the Vir plasmid in wild and laboratory strains of *E. coli* and to tag the plasmid with an antibiotic resistance marker to facilitate subsequent studies. Additional experiments were designed to (i) determine the incompatibility group to which the Vir plasmid belonged, (ii) test for the coexistence of the Vir plasmid and the ColV plasmid in the same host, (iii) determine whether the Vir plasmid could be eliminated by treatment with acridine orange, (iv) determine the molecular weight and copy number of the Vir plasmid, and (v) perform DNA-DNA hybridization experiments and DNA restriction enzyme analysis among various Vir plasmids to determine sequence homology.

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MATERIALS AND METHODS

Bacterial strains for transfer studies. The bacterial cultures used in these studies are described in Table 1. The prospective wild-type donors of the Vir character were strains JL10, JL21, and B177, and the prospective recipients were strains 711, 712, and H209. The pedigree of strains developed during the study is shown in Fig. 1 and 2.

Test for the Vir antigen. Antiserum was prepared by repeated intravenous injection of rabbits with live *E. coli* H209 Vir$^+$ and repeatedly absorbed with phenolized (0.5%) saline suspension of *E. coli* H209 until it agglutinated with the Vir$^+$ but not the Vir$^-$ form of strain H209 (26). This absorbed antiserum was used in slide agglutination tests to identify Vir$^+$ *E. coli*. There

TABLE 1. *E. coli strains used in experiments on transfer of the Vir plasmid*

Strain	Characteristics	Provided by (references):
JL10	Calf septicemia, RVC4107, serotype O78:K80:H⁻, Vir⁺[a]	P. L. Ingram (35)
JL21	Calf septicemia, Vir⁺[a]	J. Lopez-Alvarez (unpublished data)
B177	Bovine septicemia, serotype O78:K80, Col⁺ Vir⁺[a]	H. W. Smith (unpublished data)
711	K-12, F⁻ (λ) *lac-28 his-51 trp-30 proC23 phe nalA*	H. W. Smith (23)
712	K-12, F⁻ *lac rpsL*	H. W. Smith (29)
H209	Nal^r Sm^r, human fecal isolate, serotype O9:K31:H14	H. W. Smith (26)
C600(pML21)	K-12, F⁻ *thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ⁻, plasmid pML21	P. Shipley (3)
ED2149	*nia gal att* λ *bio tsx*, derivative of ED395, plasmid JCFL41	N. Willetts (37, 38)
3ΔSEX	Host for nonconjugative Sm^r Su^r plasmid	S. Falkow (11)
PACRM	K-12, F⁻ Ap^r	H. W. Smith (28)
PA10	PACRM, F Tc^r (F1 incompatibility group)	C. L. Gyles (unpublished data)
P86	Host for conjugative plasmid pCG86 Tc^r Sm^r Su^r, LT⁺ ST⁺	C. L. Gyles (15)
SF185	K-12, F⁻ Nal^r derivative of W4185	P. Shipley (25)
SF800	K-12, Nal^r derivative of p3478, *polA* mutant derivative of W3110 *thy*	P. Shipley (17)
P307	Porcine, Ent⁺	C. L. Gyles (28)

[a] Vir⁺ indicates production of the Vir toxin as well as possession of the Vir surface antigen. Neither transferability nor physical evidence for the Vir plasmid had been demonstrated for these Vir⁺ *E. coli* strains.

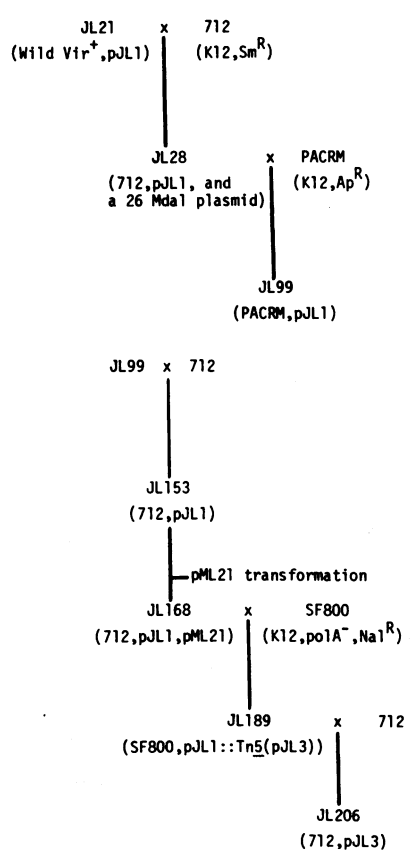

FIG. 1. *Pedigree of the E. coli strains derived as a result of transfer of the Vir plasmids pJL1 and pJL3.*

was no agglutination of any of the *E. coli* K-12 strains used as recipients in this study.

Test for the Vir toxin. Details of the test for Vir toxin have been published previously (26). A 0.2-ml volume of sterile lysate, produced by filtration of a centrifuged sonic extract of a standardized suspension of the bacteria, was injected intravenously into 3-week-old broiler chicks. Three birds were used for each test and the test was duplicated. With positive lysates all chicks died within 24 h and showed characteristic lesions (34, 35). With negative preparations there were no deaths.

Mating procedure. The mating method was similar to that described by Smith (26) for transfer of the Vir plasmid. Donor cells were eliminated from the mating mixture by two serial passages of 0.02-ml volumes of overnight broth culture through 10-ml volumes of broth containing a suitable selective antibacterial drug. The surviving cells were subcultured into antibiotic-free broth. Dilutions of the passaged mating mixture were plated on MacConkey agar, and isolated colonies were purified and then tested by slide agglutination for acquisition of the Vir antigen. In most matings, differences in the Lac phenotype and in the antibiotic resistance of donor and recipient served as aids in the identification of transconjugants.

Detection of transfer factors. Transfer factor activity was detected by the resistance determinant mobilization test of Anderson (1). The intermediate donor strain was 3ΔSEX, containing a small nonconjugative Sm^r Su^r plasmid, and the final recipient was strain 711, which is Lac⁻ Nal^r.

Mobilization of Vir. Two conjugative plasmids were used in attempts to mobilize Vir from strains JL10 and B177. One was the F plasmid with genes for tetracycline resistance (F Tc^r) carried in *E. coli* PA10 (28). The other was plasmid pCG86 (15). When strain B177 was used as a donor, transconjugants that had

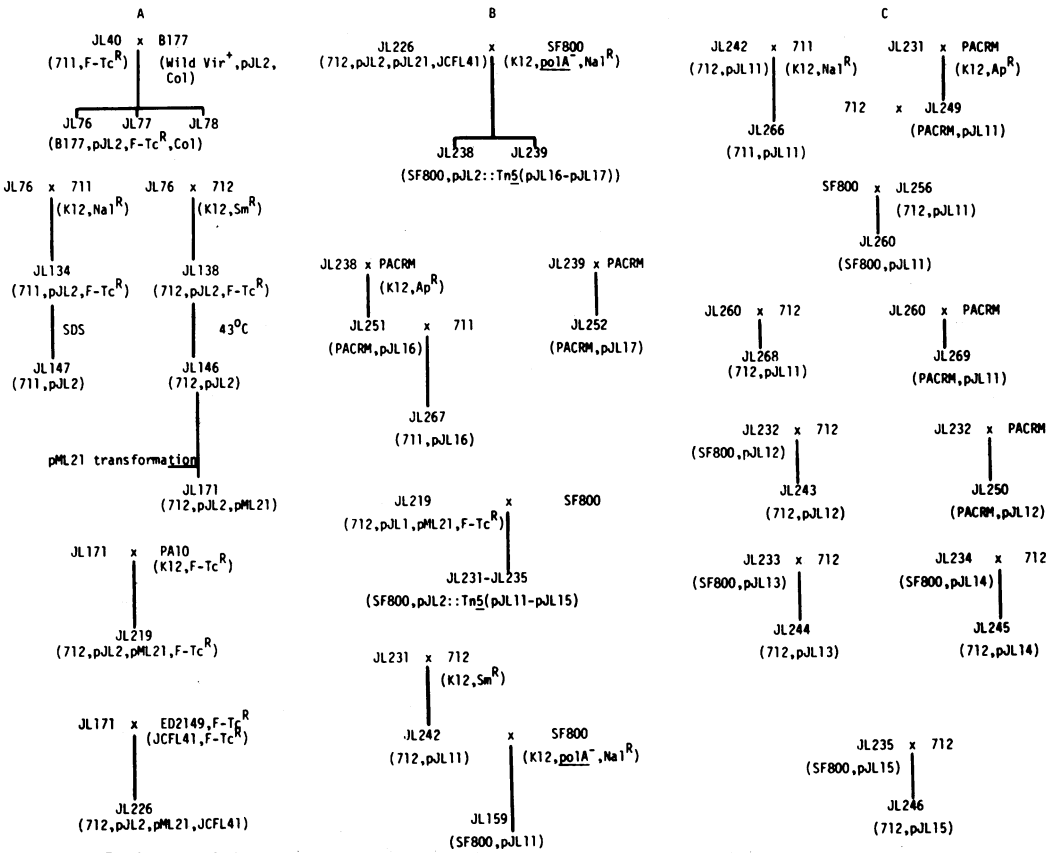

FIG. 2. *Pedigree of the E. coli strains derived as a result of transfer of the Vir plasmid pJL2 and Tn5-tagged pJL2 plasmids pJL11-pJL17. The figure is presented in three parts: A, B, and C.*

received Tc^r were tested for acquisition of Vir and colicinogeny (B177 produces a colicin which has not been typed). Colicin activity was detected by an agar overlay method (20). F Tc^r was eliminated from certain Vir^+ transconjugants by incubation of cultures at 43°C (32) or by treatment with sodium dodecyl sulfate (33).

Tagging the Vir plasmid with Tn*5* (kanamycin resistance). Plasmid pML21 (3) is a ColE1 plasmid which has been modified by integration of Tn*5* and by loss of its ability to specify production of colicin E1. Purified DNA of plasmid pML21 was produced by preparing a cleared lysate (10) of *E. coli* C600(pML21) followed by isopycnic centrifugation of the cleared lysate in a cesium chloride-ethidium bromide gradient (23). The pML21 DNA was used to transform Vir^+ cells to Kan^r and immunity to colicin E1 (7). Vir plasmids were tagged by transposition of Tn*5* from coresident plasmid pML21 (31). The transformed cells were mated with *E. coli* SF800, a *polA* mutant. Plasmid pML21 cannot replicate in the absence of DNA polymerase I (11) and is lost on transfer to the *polA* mutant; therefore, selection for strain SF800 Kan^r transconjugants will yield clones which acquired a Tn*5* insertion mutant Vir plasmid (Fig. 1 and 2). Individual transconjugant clones were tested for acquisition of the Vir antigen and toxin as well as absence of immunity to colicin E1.

Bacterial strains and phages for incompatibility studies. *E. coli* strains JL21, B177, PA10, 711, and 712 are described in Table 1. The other *E. coli* cultures and phages used in the incompatibility studies are described in Tables 2 and 6.

Incompatibility grouping of the Vir plasmid pJL1. Tests for plaquing by donor-specific phages were carried out by the method of Miller (20). The phage titer amplification test was conducted as reported by Grindley and Anderson (14).

Incompatibility grouping of Vir plasmid pJL1 was determined as follows. *E. coli* JL74, a MA335(pJL1^+) derivative which is Spc^r, was used as recipient for the incoming plasmids of the various incompatibility groups. Mating cultures were incubated for 3 h at 37°C, and dilutions were plated on spectinomycin-containing (20 μg/ml) MacConkey agar. If selection was being made for F Tc^r or R124 transconjugant recipients, tetracycline was also included at 8 μg/ml. When screening for ColB-K98^+ transconjugants was necessary, 100 recipient clones were tested for colicinogeny. Five transconjugant clones from each mating, which were considered "doubles," in that both plasmid phenotypes were expressed (the Vir phenotype and the phenotype from the incoming plasmid), were chosen for incompatibility studies. Each double clone was diluted in 0.05 M sodium phosphate buffer (pH 7.4)

and plated on MacConkey agar to obtain isolated colonies. A total of 50 colonies of each original clone was then transferred to MacConkey agar and incubated overnight at 37°C. These subcultures in antibiotic-free medium eliminated selection pressure and allowed expression of incompatibility between the two plasmids. The colonies on MacConkey agar were replica-plated onto tetracycline-containing MacConkey agar to check for the maintenance of the resistance plasmid (F Tc^r or R124) and onto plain LTC plates to check for the maintenance of the Vir plasmid or the ColB-K98 plasmid, or both, in each individual clone. Loss of either of the two plasmids at frequencies greater than those observed with the individual plasmids in separate clones was considered to be due to incompatibility.

Fertility inhibition by Vir plasmids pJL1 and pJL2. Fertility inhibition was assessed either by the repression of synthesis of conjugative pili or by a reduction in the transfer frequency of the plasmid. Repression of F-pilus synthesis by F Tc^r-containing cells, in the presence of Vir plasmid pJL2, was assessed by testing for the ability of pJL2$^+$ and pJL2$^-$ strains (JL138 and JL145, respectively) to plaque the F-specific phage fd.

The effect of the Vir plasmids on the transfer frequency of F Tc^r was studied by first introducing F Tc^r into Vir$^+$ and Vir$^-$ isogenic strains. To achieve this, *E. coli* strain PA10 was mated with isogenic Vir$^+$ and Vir$^-$ pairs of strains, 712 and JL28 and MA335 and JL74. Dilutions of the mating mixture were plated on MacConkey agar containing streptomycin (30 μg/ml) or spectinomycin (30 μg/ml) and tetracycline (8 μg/ml). F Tc^r transconjugant clones were then mated with strain PACRM to determine the effect of Vir plasmid pJL1 on the frequency of transfer of F Tc^r.

Strains JL103 and JL106 are 711 isogenic F Tc^r organisms except for the presence (strain JL103) or absence (strain JL106) of Vir plasmid pJL2. These strains were mated with *E. coli* 712 to test the effect of pJL2 on the transfer frequency of F Tc^r.

Coexistence of the Vir plasmid with the ColV plasmid. *E. coli* CA-7, kindly provided by P. Fredericq, Liege, produces colicin V1. Strain JL205 is an Sm^r mutant of strain CA-7. *E. coli* strain JL189 is an SF800 pJL3$^+$ derivative. Plasmid pJL3 is a Tn*5* insertion mutant of Vir plasmid pJL1. *E. coli* 711 is described in Table 1.

After mating of JL189 and JL205 strains, the mixture was plated on MacConkey agar containing streptomycin and kanamycin (25 μg/ml) to select for JL205 transconjugant clones which had received the Tn*5*-tagged plasmid pJL3. Fifty Kan^r Sm^r clones were replica-plated onto LTC plates (20) and then once more onto LTC and MacConkey agar containing streptomycin and kanamycin. The LTC replicas were used to determine colicinogeny and Vir status (slide agglutination) of the clones, and the kanamycin-streptomycin-MacConkey agar was used to confirm the presence of both resistance markers. With strains JL220 (K-12 ColV1$^+$ Vir$^+$ Kan^r) and 711 (Nal^r), matings were carried out for 24 h, and the mixtures were plated on MacConkey agar containing nalidixic acid and kanamycin (25 μg/ml each).

When self-transmissibility of Vir could not be detected, the plasmid was mobilized (Fig. 2A and B) either by F Tc^r or by JCFL41, a *traI* missense mutant of F'Lac, carried by *E. coli* ED2149. The *traI* gene is necessary for conjugal metabolism (36, 37), and mutations in this gene render F nontransmissible; however, sex pilus synthesis continues and the *traI* F plasmid can mobilize other plasmids in the same cell.

Transfer of Tn*5*-tagged plasmids. Matings were carried out to study the transfer of Tn*5*-tagged Vir plasmids (Fig. 2C). Members of each mating pair were grown separately as controls for the rate of mutation to resistance against the selective agent(s) included in the agar medium. Dilutions of mating mixtures and control cultures were plated (0.1 ml) in duplicate on antibiotic-containing (25 μg/ml) and plain MacConkey agar. These cultures were used to estimate the numbers of transconjugants and donor and recipient cells per milliliter in the mating mixture and to determine the rate of mutation to resistance of the organisms toward the drugs used in the plating media. The matings that were carried out are shown in Fig. 2C.

Molecular characterization of the Vir plasmids. *E. coli* strains JL10, JL21, B177, 712, H209, and PACRM are described in Table 1. Strains RG1910, JL28, and H209 Vir$^+$ are described in Table 2. Additional bacterial strains used in these experiments are characterized in Table 3. The following plasmids were used as molecular weight standards (8, 12): R1*drd-19* (62×10^6), PR4 (34×10^6), S-a (23×10^6), and RSF1030 (5.5×10^6).

Crude lysates were prepared and agarose gel electrophoresis was carried out by the methods of Meyers et al. (19). Plasmid DNA was purified as described previously. Electrophoresis of single-colony lysates was carried out essentially as described by Barnes (2). Plasmid molecular weight was calculated by comparing the extent of DNA migration in agarose gel of the plasmid in question with that of plasmid DNAs used as molecular weight standards (22).

Plasmid copy number was determined by collecting fractions of labeled DNA after isopycnic centrifugation of a cleared lysate in a cesium chloride-ethidium bromide gradient. The plasmid copy number was given by the amount of radioactivity of the plasmid peaks relative to that of the chromosomal peak, assuming a chromosome molecular weight of 2.5×10^9.

The degree of polynucleotide sequence homology of the Vir plasmids with each other and between the Vir plasmids and R124 was assayed by the use of the single-strand-specific S1 nuclease of *Aspergillus oryzae* by the method of Crosa et al. (8).

Restriction enzyme analysis of both Vir plasmids was conducted as follows. DNA of the plasmids pJL1 and pJL2 was prepared by isopycnic centrifugation of cleared lysates in cesium chloride-ethidium bromide gradients. The plasmid bands from three lysates were pooled and run through another cesium chloride-ethidium bromide gradient. These plasmid DNA samples gave a prominent band when 5 μl was electrophoresed in an agarose gel. Plasmid DNA fragments were generated with *Hin*dIII (New England Biolabs) and *Bam*HI (Miles Research Products) restriction endonucleases by digesting 15 μl of DNA in a total volume of 30 μl (5), and they were separated by electrophoresis in 0.7% agarose.

TABLE 2. *E. coli cultures and phages used in incompatibility studies with the Vir plasmids*

Culture or phage	Characteristics	Provided by (references):
Culture		
RG212	Rif^r, *his cys* λ^- derivative of *E. coli* K-12 Hfr6	R. B. Grant (unpublished data)
RG1910	*E. coli* C, R124$^+$ (Tc^r, FIV incompatibility group), Nal^r	R. B. Grant (9, 13)
J53-1	Host for Col B-K98 (FIII incompatibility group)	R. B. Grant (13, 17)
MA335	*E. coli* K-12, *pro his trp met rpsL$^+$ rpsE*	W. K. Maas (24)
JL74	MA335, Vir$^+$(pJL1) Lac$^-$ Spc^r	This study
JL103	711, Vir$^+$(pJL2), F Tc^r Col$^+$	This study
JL106	711, F Tc^r Col$^+$	This study
JL28	712 Vir$^+$(pJL1)	This study
JL138	712, Vir$^+$(pJL2) F Tc^r	This study
JL145	712, F Tc^r	This study
H209 Vir$^+$	H209(Vir$^+$), Vir derived from ovine *E. coli* S5	H. W. Smith (26)
Phage		
fd	Filamentous, F-specific DNA phage	R. B. Grant (18)
μ2	Isometric, F-specific RNA phage	R. B. Grant (21)
UA1	Isometric, FV-specific RNA phage	R. B. Grant (2)

TABLE 3. *Additional E. coli strains used in the experiments on molecular characterization of the Vir plasmids*

Strain	Characteristics
C600	K-12, F$^-$, *thr-1 leu-6 thi-1 supE44 lacY1 tonA21* λ^-; host for pBR313 (reference 23)
JL29	H209(pJL1$^+$) (JL28)[a]
JL95	JL29 Vir$^-$, after acridine orange treatment
JL99	PACRM(pJL1$^+$) (JL93)[a]
JL146	712(pJL2$^+$) (JL176)[a]
JL153-JL156	712(pJL1$^+$) (JL99)[a]

[a] Donor of the Vir plasmid.

RESULTS

Transfer of Vir from strains JL10, JL21, and B177. By mating strains JL10, JL21, and B177 with strain 712, transfer of the Vir antigen was observed only from JL21. With JL21 as the donor, 2 of 34 recipient cells acquired the Vir antigen, whereas no recipient clones from matings with JL10 (0/67) and B177 (0/104) were positive for the Vir antigen. Strains JL27 and JL28, which were 712 Vir$^+$ clones derived from matings with JL21, were tested for toxigenicity in the chick lethality assay, along with strain 712 and a 712 Vir$^-$ clone obtained from the JL21 × 712 mating. Only JL27 and JL28 were toxigenic, indicating that these transconjugants had acquired the plasmid coding for the Vir antigen and toxin. This plasmid was called pJL1 (Fig. 1).

In matings involving strain JL28 and recipient strains 711 and H209, a higher transfer frequency of Vir plasmid pJL1 was observed: 67% (28/42) of the 711 cells and 100% of the H209 cells received plasmid pJL1. Since it proved to be an excellent recipient for pJL1, strain H209 was used in matings with JL10 and B177, the other Vir$^+$ wild strains. Neither JL10 (0/102) nor B177 (0/109) transferred Vir to H209. Three Vir$^+$ and four Vir$^-$ clones of strain H209 derived from matings with JL28, and seven Vir$^-$ H209 and 712 clones derived from matings with strains JL10 and B177 were assayed for toxigenicity in chickens. Only those clones with the Vir antigen were toxigenic. The possibility of temperature-sensitive transfer factors in strains JL10 and B177 was explored by conducting matings of these strains with 712 at 22 and 30°C, but no transfer of Vir was detected.

Transfer factors in the toxigenic strains. Vir$^+$ and Vir$^-$ toxigenic strains were tested for the presence of transfer factors capable of mobilizing the small nonconjugative Sm^r Su^r plasmid (Table 4). All Vir$^+$ strains except JL10 showed transfer factor activity. The frequency of cotransfer of Vir was very high for strains JL21, JL27, and JL28, but Vir was not transferred from B177. Matings with strains JL21, JL27, and JL28 (all containing the same Vir plasmid) yielded Lac$^+$ Sm^r Nal^r clones in addition to the "normal" Lac$^-$ Sm^r Nal^r 711 transconjugant clones (Table 4). These clones did not represent mutant clones of any of the three members of a mating since the rate of mutation of these strains to either Lac$^+$ (711) or Nal^r (JL21, JL27, JL28, and 3ΔSEX) must be of the order of $<10^{-8}$ as evidenced by experiments performed to rule out this possibility.

Mobilization of Vir from strains JL10 and B177. The only successful mobilization of a Vir plasmid from the two wild strains JL10 and B177 occurred with use of the F Tc^r plasmid to transfer Vir from strain B177 into 711 (Table 5). This

TABLE 4. *Transfer factor activity of Vir⁺ E. coli strains as detected by mobilization of a nonconjugative Sm^r Su^r plasmid into E. coli K-12*

Donor	Characteristics	711 Sm^r transconjugants (Lac⁻) in mating mixture[a] (per 0.2 ml)	Cotransfer of Vir and Sm^r Su^r[b]
JL10	Wild Vir⁺	0	0/0
JL21	Wild Vir⁺	400[c]	18/20 (90%)
JL27	712 Vir⁺[d]	40[c]	10/10 (100%)
JL28	712 Vir⁺[d]	30[c]	9/10 (90%)
B177	Wild Vir⁺	400	0/50 (0%)

[a] The final recipient, 711, is a K-12 Lac⁻ Nal^r organism. All donors are Lac⁺. The intermediate strain, 3ΔSEX, is a Lac⁺ *E. coli* with the nonconjugative Sm^r Su^r plasmid. Selection was made on MacConkey agar containing nalidixic acid (25 μg/ml) and streptomycin (25 μg/ml) and permitted the recovery of final-recipient (711) clones into which the Sm^r Su^r plasmid had been mobilized by the donor. *E. coli* P307 (see Table 1) was used as a positive control strain for transfer factor activity.

[b] Values represent number positive per number tested.

[c] Lac⁺ Sm^r Su^r *E. coli* 711 clones were also recovered from these matings: 1, 28, and 22 clones per 0.2 ml from the matings involving JL21, JL27, and JL28, respectively.

[d] The Vir plasmid was acquired from *E. coli* JL21.

TABLE 5. *Mobilization of the pJL2 plasmid from three B177(F Tc^r) clones into E. coli K-12*

Matings[a]	711 F Tc^r transconjugants per ml[b]	F Tc^r transfer frequency per donor cell	Cotransfer of pJL[2] and F Tc^r [Vir + 711(F Tc^r) clones]
JL76 × 711	1,500	10^{-6}	1/40 (2.5%)
JL77 × 711	50	5×10^{-8}	3/8 (37.5%)
JL78 × 711	450	5×10^{-7}	4/40 (10%)

[a] The donors JL76, JL77, and JL78 were produced by transferring the F Tc^r plasmid from *E. coli* strain PA10 (Table 1) into *E. coli* B177.

[b] Donor and recipient cultures were mated at 37°C for 24 h, and transconjugants were selected on MacConkey agar containing nalidixic acid (25 μg/ml) and tetracycline (25 μg/ml). The donors carry Tc^r, and the recipient is Nal^r.

Vir plasmid was named pJL2. One pJL2 F Tc^r clone (JL134) and one 712(pJL2 F Tc^r) clone (JL138) were cured of F Tc^r to obtain clones containing the pJL2 plasmid only (Fig. 2A). Neither incubation at 43°C nor treatment with 10% (wt/vol) sodium dodecyl sulfate (33) eliminated pJL2 from these strains (100 clones tested).

Tagging of pJL1 and pJL2 with Tn*5*. *E. coli* strains JL153 and JL146, which are 712 derivatives containing pJL1 and pJL2, respectively, as the only resident plasmid, were first transformed with pML21 DNA (Fig. 1 and 2A). Strain JL153 was transformed to Kan^r at a frequency of 5×10^{-6} per viable cell per μg of pML21 DNA. JL146 was transformed to Kan^r at a frequency of 1.5×10^{-5} per cell per μg of pML21 DNA. Twelve transformant clones of each strain were streaked on colicin E1-containing LB plates; all were resistant to this colicin, confirming the acquisition of pML21.

When *E. coli* strains JL168 and JL171 (transformant clones derived from JL153 and JL146, respectively) were mated with *E. coli* SF800, Kan^r transconjugants were obtained only from the mating with strain JL168. Four of these Kan^r transconjugants were tested by slide agglutination in Vir antiserum. All four were Vir⁺, indicating that pJL1 had been tagged with Tn*5*. The plasmid pJL1::Tn*5* from one of these clones (JL189) was named pJL3 (Fig. 1).

Since conjugal transfer of Tn*5*-tagged pJL2 was not detected, mobilization of this plasmid by JCFL41 and F Tc^r was attempted. *E. coli* strain JL219, a JL171(F Tc^r) derivative (Fig. 2B), was used as donor in matings with *E. coli* SF800. Kan^r was transferred at a frequency of 2.4×10^{5} per donor cell, and 27% of the transconjugants (100 colonies tested) were F Tc^r Vir⁺, indicating a pJL2::Tn*5* transfer frequency of at least 6.4×10^{-6} per donor. To mobilize Tn*5*-tagged pJL2 with JCFL41, *E. coli* strain JL226, a JL171 (JCFL41) clone (Fig. 2B), was used as donor in matings with *E. coli* SF800. Kan^r was transferred at a frequency of 1.8×10^{-7} per donor cell. Two SF800 Vir⁺(pJL2::Tn*5*) (JL226) transconjugants were named JL238 and JL239.

Transfer of the Tn*5*-tagged Vir plasmids. In previous experiments, pJL1 behaved as a derepressed plasmid, and pJL2 was either a nonconjugative or a repressed plasmid whose transfer was not detectable in the absence of a selection marker in the plasmid. The pJL1::Tn*5* plasmid transferred at high frequencies from strain JL206 (Fig. 1) into *E. coli* SF185. The transfer frequency per donor cell ranged from 2×10^{-5} after 5 min of mating to 8.6×10^{-2} after 60 min of mating. The tagged pJL2 plasmids were found to be conjugative and to transfer at various frequencies, ranging from 5.6×10^{-6} to 1.3×10^{2} in 24-h matings.

Sensitivity to the F-specific phages fd and μ*2*. Vir plasmid pJL1 conferred sensitivity to phages fd and μ2 (Table 6), provided it was not accompanied by one of the two cryptic plasmids originally present in the wild bovine septicemic strain JL21, from which it originated. The other Vir plasmid, pJL2, did not confer sensitivity to plaquing by these phages, unless the trans-

TABLE 6. *Sensitivity to the F-specific phages fd and μ2*

Strain	Characteristics	Plaqued by:[a]	
		fd	μ2
712, PACRM SF800, MA335	K-12	−	−
RG212	K-12 Hfr6	++++	++++
JL10	Wild Vir$^+$, no plasmids	−	−
H209 Vir$^+$	H209 Vir$^+$ (S5)[b] (Smith, 1974), at least three plasmids	−	−
JL29	H209(pJL1$^+$), at least four plasmids	++	++
JL21	Wild pJL1$^+$ and two cryptic plasmids	−	−
JL28	712(pJL1$^+$) and one cryptic plasmid (JL21)	++	++
JL153	712(pJL1$^+$) (JL99)[b]	+++	+++
JL99	PACRM(pJL1$^+$) (JL93)[b]	++	++
B177	Bovine septicemic, pJL2$^+$, Col$^+$	−	−
JL146	712(pJL2$^+$) (JL76)[b]	−	−
JL171	JL146(pML21$^+$)	−	−
JL231	SF800(pJL11$^+$)[c] (JL219)[b]	−	−
JL242	712(pJL11$^+$) (JL231)[b]	++	++
JL249	PACRM(pJL11$^+$) (JL231)[b]	++	+
JL256	712(pJL11$^+$) (JL249)[b]	+	++
JL260	SF800(pJL11$^+$)	++++	++++

[a] ++++, Efficiency of plating (EOP) >0.1 to 1.0; +++, EOP >0.01 to <0.1; ++, EOP >0.001 to >.01; +, EOP >0.0001 to <0.001; EOP >10^{-4}.
[b] Donor of the Vir plasmid.
[c] A pJL2::Tn*5* plasmid.

poson Tn*5* was inserted in it (plasmid pJL11 = pJL2::Tn*5*).

Strains JL10, H209 Vir$^+$, H209, and B177 were tested for phage fd titer amplification. With *E. coli* RG212 as indicator strain, the initial titer of the fd phage suspension was determined. A 0.1-ml volume of phage suspension was added to 1 ml of a fresh broth suspension of the test strain, and the mixture was incubated overnight at 37°C. The cultures were centrifuged, and the supernatant was diluted 10-fold up to 10^{-10} in diluent buffer. The final titer of the supernatant was determined by using *E. coli* RG212 as the indicator strain. Strain H209 Vir$^+$ amplified the fd phage titer by a factor of 16,000-fold higher than that of the background, whereas strains H209, JL10, and B177 had very little effect on the fd titer. The sensitivity to the F-specific phages conferred by the pJL1 and pJL2 plasmids placed them as members of the F-incompatibility complex.

Incompatibility grouping of pJL1. F Tc^r was implanted in strain JL74 [an *E. coli* K-12(pJL1$^+$); Table 7] with an efficiency of 1.2 per PA10 donor cell. The rate of transfer of the ColB-K98 plasmid into strain JL74 was of the order of 0.5 per J53-1 donor cell. R124 was transferred at a rate of 8×10^{-4} per RG1910 donor cell into strain JL74. The incompatibility reactions between these incoming plasmids and

TABLE 7. *Incompatibility reactions between the Vir plasmid pJL1 and incoming plasmids of the F-incompatibility complex in strain JL74*

Transconjugant clones[a]	Frequency[b] of elimination of:	
	Incoming plasmid	pJL1
JL74 (F Tc^r) (PA10) 1-5	0 (FI)[c]	0
JL74(ColB-K98$^+$) (J53-1) 1-5	0 (FIII)	0
JL74, R124$^+$ (RG1910)		
1	20	0
2	17	1
3	6 (FIV)	38
4	13	6
5	22	1

[a] Five clones of *E. coli* JL74 [K-12(pJL1$^+$)] which had received the F Tc^r plasmid from *E. coli* PA10; five clones which had received the ColB-K98 plasmid from *E. coli* J53-1; and five clones which had received the R124 plasmid were selected.
[b] Each clone was diluted in 0.05 M sodium phosphate buffer and plated on MacConkey agar to produce isolated colonies. Fifty colonies derived from each clone were transferred to MacConkey agar and incubated and then replica-plated to determine how many maintained the F Tc^r, the ColB-K98, or the R124 plasmid. For each of the three plasmids, a total of 250 colonies was examined.
[c] The incompatibility group of the incoming plasmid.

pJL1 are summarized in Table 7, which shows that pJL1 was incompatible with R124 and either of the two plasmids was eliminated from the "doubles." Most commonly, R124, which was the incoming plasmid, was eliminated. Strains JL28 and JL99 were insensitive to plaquing by phage UA1.

Fertility inhibition characteristics of pJL1 and pJL2. The rates of transfer of F Tc^r from strain PA10 to two different pJL1$^+$ and pJL1$^-$ homogenic pairs of strains and from these strains in turn to strain PACRM were compared. The presence of pJL1 did not cause exclusion of the entry of F Tc^r, nor did pJL1 inhibit transfer of F Tc^r when both plasmids were present in the same cell. Plasmid pJL1 therefore behaved as an fi$^-$ plasmid or a derepressed fi$^+$ plasmid.

E. coli strain JL103 [a 711(pJL2$^+$, F Tc^r, Col$^+$) derivative] transferred F Tc^r to strain 712 at a frequency of 2×10^{-2} per donor cell, whereas the pJL2$^-$ homogenic strain JL106 transferred at a frequency of 2.4×10^{1}. This represents a 1,200-fold difference in the rate of transfer of F Tc^r, indicating a fertility inhibition effect of pJL2 on F Tc^r.

Plasmid pJL2 repressed sex pilus synthesis in the 712(F Tc^r, pJL2$^+$) strain JL138, inhibiting plaquing of this strain by phage fd. This phage forms plaques on (+++) the pJL2$^-$ isogenic organism, strain JL145.

Coexistence of Vir and ColV plasmids. When JL189 [SF800(pJL3$^+$)] was mated with JL205 Sm^r(ColV1$^+$), 46 of 50 Kan^r JL205 transconjugant clones were ColV1$^+$ Vir$^-$, two were ColV1$^+$ Vir$^+$, and two were ColV1$^-$ Vir$^+$. When a JL205 ColV1$^+$ Vir$^+$ clone was mated with *E. coli* 711, Kan^r was transferred to 711 with a frequency of 5×10^{-8} per donor cell, and 18% of the 711 transconjugants were ColV1$^+$. Only those clones expressing the ColV1$^+$ character were Vir$^+$; none of the ColV$^-$ clones was Vir$^+$.

Acridine orange treatment of Vir$^+$ cells. The following cultures were subjected to acridine orange treatment (20): JL21, a wild Vir$^+$ *E. coli* from which pJL1 originated; JL27 and JL28, both 712(pJL1$^+$) transconjugants; JL29, an H209(pJL1$^+$) derivative; JL60, a 711(pJL1$^+$) transconjugant; SF800(pJL1$^+$) and B177, the original host strain for pJL2. The culture tubes with the highest concentration of acridine orange (125 μg/ml) were selected in screening for cured cells. None of the 50 clones tested from each strain had lost the Vir plasmid, except for one Vir$^-$ clone recovered from the JL29 [H209(pJL1$^+$)] culture. This clone was negative for the Vir antigen by slide agglutination and was not toxigenic for chickens but plaqued the fd phage.

Molecular weight of the Vir plasmids. By electrophoresing ethanol-precipitated plasmid DNA in 0.7% agarose gels, the molecular weights of pJL1 and pJL2 were determined by estimating their mobilities relative to those of the molecular weight standards. Both Vir plasmids (Fig. 3) had identical mobilities (0.86 relative to that of R1*drd-19*), corresponding to a molecular weight of 92×10^6. In addition to pJL1, strain JL28 contained a smaller plasmid of molecular weight 26×10^6. The Col plasmid from strain B177 had a molecular weight of 54×10^6.

Purified plasmid DNA from strains JL99 [PACRM(pJL1$^+$)] and JL146 [712(pJL2$^+$)] showed single bands with identical mobilities, corresponding to pJL1 and pJL2, respectively (Fig. 4). No plasmid band was detected in lysates from strain JL10, nor could any plasmid band be associated with Vir in H209 Vir$^+$ (26) and JL29, which is H209 Vir$^+$ (JL28). All H209 derivatives, including JL95 (a JL29 Vir$^-$ clone produced by acridine orange treatment) had the same electrophoretic pattern. In addition, strains JL29 and JL95 contained the smaller plasmid present in strain JL28, which was transferred from this strain together with the Vir character. Strain JL21 contained pJL1 and two additional plasmids, one of which was transferred to strain JL28 along with pJL1. The molecular size of plasmid pJL1 was confirmed in

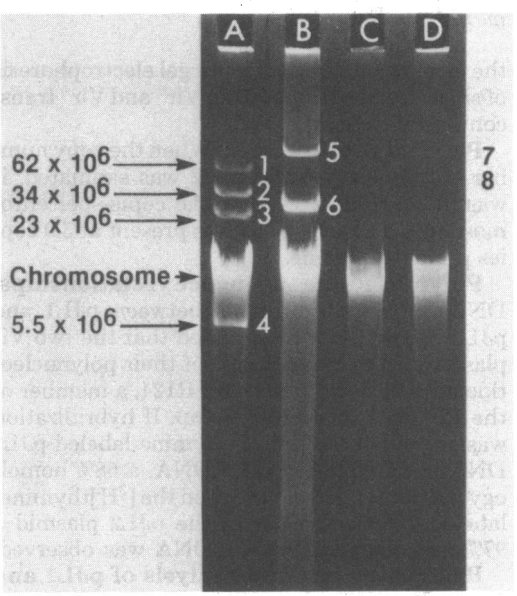

FIG. 3. *Agarose gel electrophoresis of ethanol-precipitated DNA. (A) Molecular weight standards: bands 1-4. (B) JL28 [712(pJL1$^+$)]: upper band (5) is due to plasmid pJL1; the lower band (6) represents a cryptic plasmid. (C) E. coli 712: chromosomal DNA. (D) B177; upper band (7) is due to plasmid pJL2; the second band (8) is due to a colicin plasmid.*

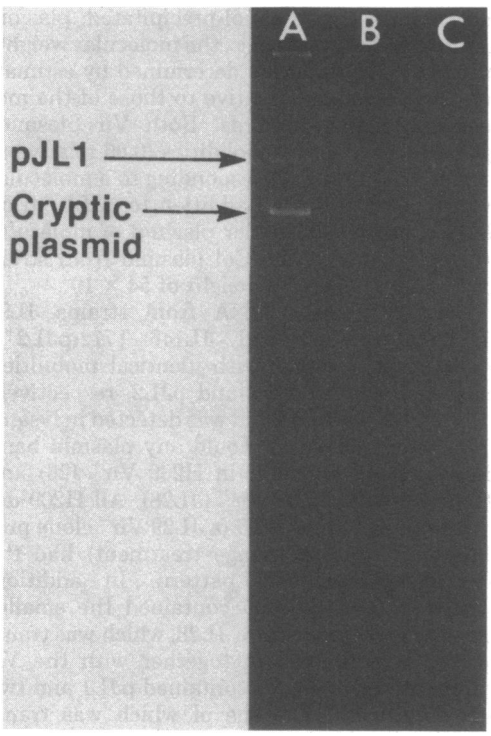

FIG. 4. *Agarose gel electrophoresis of purified plasmid DNA. (A) JL28(pJL1 and a cryptic plasmid). (B) JL99 [PACRM(pJL1$^+$)]. (C) JL146 [712 (pJL1$^+$)].*

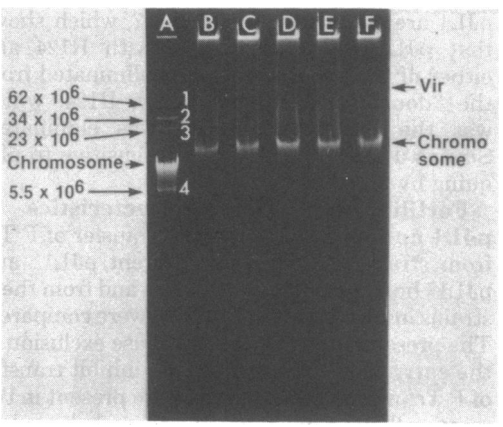

FIG. 5. *Agarose gel electrophoresis of single-colony lysates from 712(pJL1) (JL99) transconjugants (C–F). (B) Vir$^-$ 712 (JL99) transconjugant. (A) Molecular weight standards (1–4).*

the experiments with agarose gel electrophoresis of single-colony lysates from Vir$^-$ and Vir$^+$ transconjugants (Fig. 5).

Plasmid copy number. When the copy number of the Vir plasmid pJL2 was estimated, it was found to be present at 3.8 copies per chromosome. Similarly, pJL1 was present at 3.9 copies per chromosome.

Polynucleotide sequence relationships. DNA-DNA hybridizations between pJL1 and pJL2 and R124 DNA revealed that the two Vir plasmids shared about 40% of their polynucleotide sequences with plasmid R124, a member of the FIV incompatibility group. If hybridization was carried out with [^{3}H]thymine-labeled pJL1 DNA and unlabeled pJL2 DNA, a 58% homology was found. However, when the [^{3}H]thymine-labeled DNA was that of the pJL2 plasmid a 97% homology with pJL1 DNA was observed.

Restriction enzyme analysis of pJL1 and pJL2. Both Vir plasmids were digested by the restriction endonucleases *Hin*dIII and *Bam*HI. From analysis of the restriction endonuclease digestion patterns of both plasmids by *Bam*HI, it was evident that they were different (Fig. 6). *Bam*HI enzyme cleaved pJL1 DNA at seven different sites to produce seven fragments,

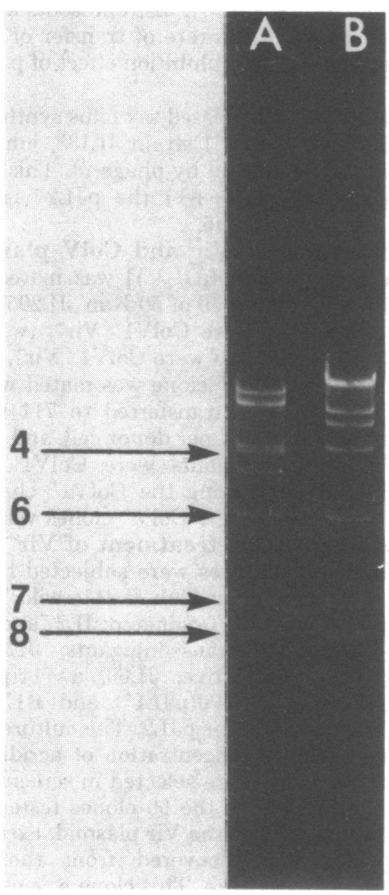

FIG. 6. *Pattern of bands obtained after digestion of plasmids pJL2 (A) and pJL1 (B) with the restriction endonuclease BamHI. The bands labeled 4, 6, 7, and 8 appear to be DNA fragments of the same size.*

whereas the enzyme cleaved pJL2 DNA into eight fragments. Four of the *Bam*HI-generated fragments from both plasmids were of the same molecular weight. *Hin*dIII, on the other hand, generated 15 fragments from pJL1 and 18 fragments from pJL2 DNA. Of the fragments produced by *Hin*dIII from both plasmids, eight seemed to be of identical molecular weights, but the large number of closely spaced bands prevented accurate analysis.

DISCUSSION

In 1974, Smith (26) reported his discovery of the Vir plasmid in an *E. coli* recovered from bacteremic disease in a lamb. That the Vir phenotype was determined by a plasmid was inferred from the transferability of the Vir characteristics in mating experiments and from the fi^+ behavior of the transfer factor associated with Vir. In the present study, the genetic element coding for Vir in three wild *E. coli* isolates and one laboratory-derived Vir^+ *E. coli* produced by Smith (26) was investigated. A plasmid responsible for the Vir character in two wild *E. coli* strains was demonstrated not only by transfer experiments but also by isolation of plasmid DNA.

The transfer characteristics of the two plasmids from the wild strains were markedly different. One Vir plasmid, pJL1, was transferred at a high rate from its wild host and at an even higher rate from $pJL1^+$ transconjugants that had not received an fi^+ plasmid which was present in the parent strain. Plasmid pJL1 behaved in transfer experiments like a derepressed plasmid. The second Vir plasmid, pJL2 (derived from *E. coli* B177), was not transferred at a frequency high enough to be detected in mating experiments with no selective device but was mobilized by means of a transmissible plasmid. Vir was not transferred from the third wild *E. coli*, nor was any plasmid detected on agarose gel electrophoresis of DNA from this organism. It is likely that the Vir character is determined by chromosomal genes in this *E. coli* strain.

The stability of both Vir plasmids in *E. coli* was evidenced by failure of treatment with sodium dodecyl sulfate or incubation at 43°C to eliminate plasmid pJL2 and failure of acridine orange treatment to eliminate either pJL1 or pJL2. This failure is consistent with the previous report (26) of unsuccessful attempts to eliminate the Vir plasmid by acridine orange treatment.

When the pJL2::Tn*5* plasmids were mobilized by the *traI* F'Lac mutant plasmid JCFL41 into strain SF800, 25% of the Kan^r transconjugant clones which lacked the Vir antigen points to a possible insertion of Tn*5* in the Vir antigen gene(s). The possibility that JCFL41::Tn*5* was mobilized by pJL2 from the donor into SF800 is remote, since control experiments demonstrated that JCFL41 either is not transferred from *E. coli* JL226 or is transferred at a frequency less than 2×10^{-4} per donor cell.

The striking differences in the frequency of transfer of pJL2::Tn*5* plasmids is strange. It is possible that Tn*5* might have inserted in a plasmid sequence which is directly or indirectly related to the control of the transfer function. The insertion of Tn*5* might interfere with the repressor mechanisms of transfer in a defective manner, allowing it to be expressed at times but not at others. However, this phenomenon was observed for two independently derived pJL2::Tn*5* plasmids, and this explanation would imply a similar insertion of Tn*5* in both cases. Although Tn*5* insertions are known to be non-site-specific, and even within a small sequence like the *lacZ* gene Tn*5* insertions are random (3, 4), it may be that Tn*5* has a preferred site on the Vir plasmid. An alternative explanation for the derepression effect of the Tn*5* insertion in pJL2 is the presence of a derepressing locus in the Tn*5* sequences. A derepressing locus for F-*lac* has been described (25) to be associated with a determinant for kanamycin resistance.

That pJL1 is a derepressed plasmid and the strains containing it plaque phages fd and $\mu 2$, which are specific for F-like pili, greatly facilitated the classification of this plasmid by incompatibility grouping. This plasmid was incompatible with R124, a member of the FIV incompatibility group. Because it is depressed, pJL1 behaved as an fi^- plasmid and did not reduce the normal frequency of transfer of F Tc^r.

The repressed plasmid pJL2 did not induce plaquing by the fd and $\mu 2$ phages in its host strain; however, insertion of the kanamycin transposon Tn*5* derepressed the plasmid for sex pilus synthesis and allowed the plaquing of its host strains by the F-specific phages. Plasmid pJL2 behaved as an fi^+ plasmid, reducing the frequency of transfer of F Tc^r by a factor of 1,200 and repressing F pilus synthesis, thereby rendering its host insensitive to plaquing by the fd phage. Plasmid pJL2 seems to code for an efficient repressor of the transfer operon, since in fd phage amplification tests cultures containing the plasmid failed to even double the titer of the phage.

Strain H209 Vir^+ harbored a repressed Vir plasmid capable of amplifying the fd titer more than 16,000-fold. This finding is consistent with that of Smith (26) that the transfer factor of the Vir plasmid was fi^+. However, the plasmid is probably inserted in the chromosome in this

strain since its presence could not be demonstrated by agarose gel electrophoresis.

The incompatibility of Vir and ColV1 plasmids probably explains the observations (26, 27, 30) that in studies involving hundreds of ColV$^+$ septicemic *E. coli* none have been found to be Vir$^+$; moreover, no Vir$^+$ isolates have been found to be ColV$^+$. Recombinants may, however, be expected to arise in nature.

Although both pJL1 and pJL2 have the same molecular weights and are present at about four copies per bacterial chromosome, they are not identical since the *Bam*HI and *Hin*dIII restriction enzyme patterns of the two plasmids are different. When the polynucleotide sequence homology between pJL1 and pJL2 was analyzed by DNA-DNA hybridization, it was found that when the hybridization reaction was carried out with [^{3}H]thymine-labeled pJL1 DNA, the homology was 58%, but when the reaction was reversed by using ^{3}H-labeled pJL2 DNA, the homology was 97%. Although the differing results might be due to technical error, the possibility of actual differences due to singular characteristics of one of the plasmids cannot be discarded. One such possibility is the presence of repeated sequences (6), either inverted or direct, in pJL2, which is the plasmid that gave a higher ^{3}H count when hybridized with cold heterologous pJL1 DNA. These repeated sequences could reassociate themselves and thereby account for a higher sequence homology value if pJL2 is the ^{3}H-labeled plasmid, but not if it is contributing unlabeled DNA.

Virulence plasmids have been detected in several animal and human pathogens (11, 16, 22, 23, 26, 27, 29, 30) as well as in a plant pathogen (39). The characterization of these plasmids and the isolation of their DNA could contribute significantly to our understanding of these diseases in which virulence factors are determined by plasmids.

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