JOURNAL OF BACTERIOLOGY, Sept. 1992, p. 5953–5960
0021-9193/92/185953-08$02.00/0

Vol. 174, No. 18

Determination of the Mechanism of Retrotransfer by Mechanistic Mathematical Modeling

EVA TOP,[1]* PETER VANROLLEGHEM,[1] MAX MERGEAY,[2] AND WILLY VERSTRAETE[1]

Laboratory of Microbial Ecology, Faculty of Agricultural Sciences, University of Gent, Coupure Links 653, B-9000 Ghent,[1] and Laboratory of Genetics and Biotechnology, Vlaamse Instelling voor Technologisch Onderzoek, Boeretang 200, B-2400 Mol,[2] Belgium

Received 18 February 1992/Accepted 2 July 1992

Two mathematical models to elucidate the mechanism of retromobilization (or retrotransfer), that is, the ability of conjugative plasmids to mobilize genes into the cell containing the conjugative plasmid, were developed. This study deals with retromobilization of nonconjugative plasmids (Tra^- Mob^+). Plasmid transfer was modeled by two mass action models. The first is based on the hypothesis that retromobilization of the Tra^- Mob^+ vector occurs in one step, by means of the pilus formed by the Tra^+ plasmid in the original host. In the second model, retromobilization is considered to be a two-step process involving two transfer events. The first step involves the transfer of the Tra^+ plasmid from the recipient cell to the donor of the nonconjugative vector, and during the second encounter the nonconjugative vector is mobilized toward the recipient. Since the relationships between the number of transconjugants and the number of recipients for the two models are different, filter matings were performed for short time periods with different initial densities of the recipient population. Comparison of the numbers of transconjugants with the results of the mathematical equations confirmed the hypothesis that retromobilization is a one-step conjugation process.

Plasmids belonging to the incompatibility groups IncC, IncJ, IncN, IncP (IncP1), IncQ, and IncW can be maintained in a wide range of bacterial species. Many of these broad-host-range plasmids are able to self-transfer and to mobilize nonconjugative (Tra^-) plasmids and sometimes even chromosomal genes into a very wide range of gram-negative bacteria (29). DNA mobilization by IncP1 plasmids toward gram-positive bacteria (7, 16, 22, 32), cyanobacteria (11, 34), and yeasts (8) has been demonstrated; the replication range of IncQ plasmids could also be extended to cyanobacteria (11), gram-positive bacteria (6), and plants (1). In addition, IncP1 (and some IncN) plasmids mobilize plasmids and chromosomal genes not only in the classical forward direction, i.e., from donor to recipient of the Tra^+ plasmid, but also in the reverse direction, i.e., from recipient to donor. This phenomenon of reverse transfer is called retrotransfer and has been observed in both homologous and intergeneric matings.

Retrotransfer of chromosomal auxotrophic markers with the IncP1 plasmid pULB113 (RP4::Mu3A) was observed in homologous matings with *Pseudomonas fluorescens*, *Salmonella typhimurium*, *Alcaligenes eutrophus*, and *Erwinia chrysanthemi*, as well as in heterospecific matings between *A. eutrophus* and *Pseudomonas putida* carrying pULB113, at frequencies similar to those of the direct mobilization (17). The results obtained in this study gave rise to the assumption that retrotransfer could be an early event in the conjugation process and so would not depend upon the stable acquisition of the conjugative plasmid by the recipient but would occur as a one-step process of bidirectional DNA transfer.

Retromobilization of a Tra^- Mob^- vector, containing the heavy metal resistance genes *czc*, from *Escherichia coli* toward *A. eutrophus* harboring pULB113 occurred by integration of the *czc* fragment in pULB113 at a frequency of 10^{-7} to 10^{-8} per recipient, which was virtually the same frequency as that of triparental mobilization (31).

Retromobilization of nonconjugative mobilizable (Tra^- Mob^+) plasmids has been demonstrated for many IncP1 plasmids (RP4, pUZ8, and two catabolic plasmids, pJP4 and pSS50) (20, 27, 28, 31). Of particular importance is the observation that the IncP1 plasmid pRK2013 is able to retromobilize plasmids from strains in which it cannot be stably maintained: pRK2013 was shown to retromobilize an IncQ vector from *Desulfovibrio* spp., which belong to the δ subgroup of the purple bacteria, toward *E. coli* (20). A few plasmids belonging to other incompatibility groups (IncN, IncM) can also retromobilize IncQ vectors (28), yet retrotransfer seems to be restricted to only a few broad-host-range plasmids, particularly those from the IncP1 group.

Retrotransfer might be important ecologically; it might be an elegant way for IncP1-bearing bacteria to capture new genetic information from other organisms. In this way, retrotransfer could help communities become adapted to changing environmental conditions (17, 18). Retrotransfer must also be considered during the assessment of the fate of released genetically engineered microorganisms and their DNA sequences into natural environments. An enhanced dissemination of genes into a microbial community could be expected when retromobilizing plasmids are present among the autochtonous bacteria in the biotope.

To predict the dissemination of genes in natural environments after introduction of an allochtonous bacterium, simulation of gene transfer and survival of the introduced DNA is required. A number of groups have studied the kinetics of direct conjugal plasmid transfer by using mathematical models. All of the models are based on a mass action approach, in which the rate of transconjugant formation is jointly proportional to the densities of donor and recipient cells. This mass action model assumes that parental cells are randomly distributed in homogeneous populations (5, 13). These models have been applied and extended for the prediction of plasmid transfer and transconjugant survival in

* Corresponding author.

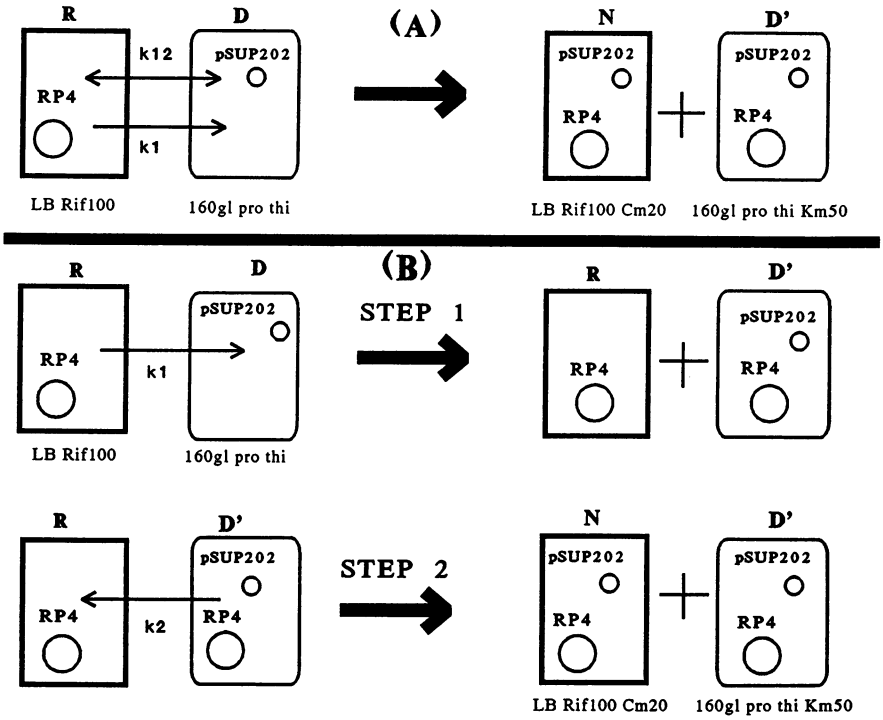


FIG. 1. Schematic representation of retromobilization of the nonconjugative (Tra^-) plasmid pSUP202 by the conjugative (Tra^+) plasmid RP4 with the one-step model (A) and the two-step model (B). *R*, recipient of pSUP202, LE392(RP4) (box); *D*, donor of pSUP202, CSH52(pSUP202) (rounded box); *D'*, donor transconjugant (donor that has received RP4); *N*, retrotransconjugant (recipient that has received pSUP202). For explanation of the *k* parameters, see the text. 160gl, mineral medium (33) with 0.5% glucose as the C source; pro, proline (40 μg/ml); thi, thiamine (40 μg/ml); Rif100, rifampin (100 μg/ml); Cm20, chloramphenicol (20 μg/ml); Km50, kanamycin (50 μg/ml).

soil microcosms, where they fitted with the experimental data (2, 10). The dynamics of plasmid transfer on surfaces and the adequacy of the model under these conditions was investigated by a method in which thin agar slides were used (24). The model has also been used for estimating the net rate of plasmid transfer in batch cultures (26) and multistage continuous cultures (30) and for studying the conditions for the establishment and maintenance of plasmids in bacterial populations (25). A model for mobilizable plasmids hitchhiking with a conjugative plasmid has been developed (12); in this model, mobilization of a nonconjugative plasmid is only possible when a conjugative plasmid is also present in the donor cell.

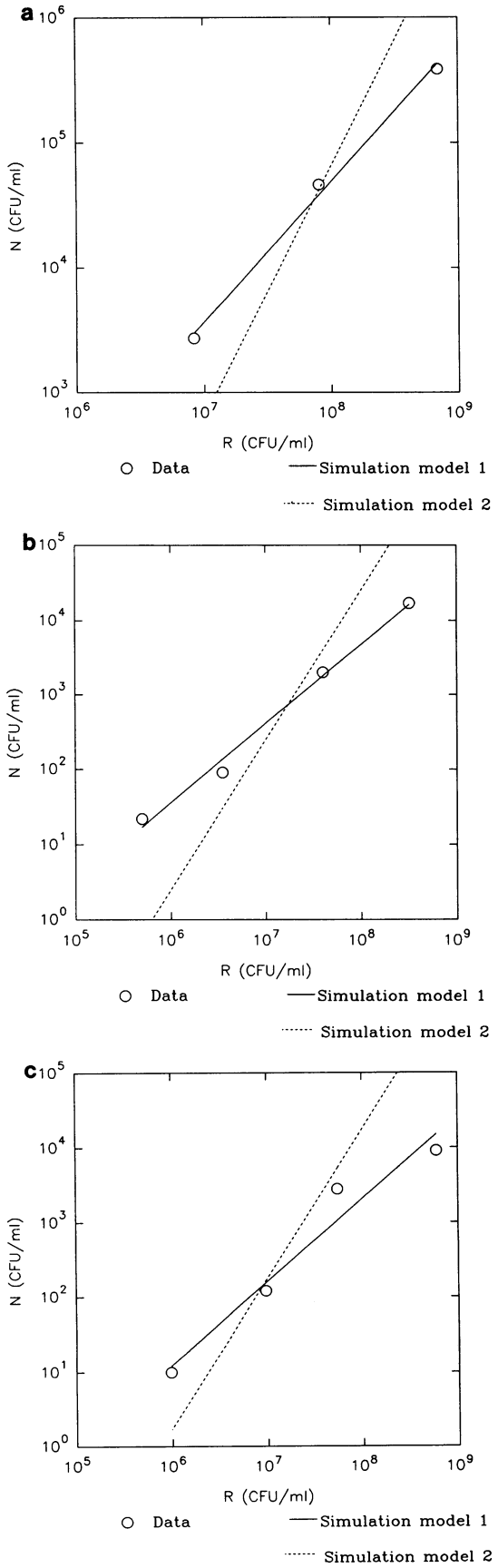
As far as we know, the kinetics of retromobilization of plasmids have not been studied. The aim of this work is to investigate whether retromobilization of nonconjugative plasmids occurs as a one-step or two-step process. In other words, is retromobilization the result of only one encounter between the cell harboring the conjugative plasmid and the cell containing the nonconjugative plasmid, during which DNA moves freely in two directions, or does the process involve two encounters, like a triparental mating? To investigate which model fits with the experimental data, two mechanistic mathematical models, based on these two different assumptions, were developed.

MATERIALS AND METHODS

Strains and plasmids. Homologous matings with a rifampin-resistant mutant of *E. coli* LE392 (*pro met*) (21), harboring the conjugative plasmid RP4 (Tc Ap Km) (3), and *E. coli* CSH52 (*pro thi*), harboring the nonconjugative vector pSUP202 (Tc Ap Cm) (23) were performed.

Media. The mating experiments were performed on Luria Bertani (LB) agar, and the cultures were grown in 5 ml of LB broth containing the appropriate antibiotics. The media used for selective enumeration of the different cell lines are described in the legend to Fig. 1.

Mating procedure. Since transfer of broad-host-range plasmids like RP4 occurs most efficiently on solid media (4), a filter mating procedure was used to assess our conjugation models. Cultures were grown overnight in LB broth supplemented with the appropriate antibiotics. Aliquots (0.5 ml) of both parental strains were mixed in an Eppendorf tube and centrifuged for 2 to 3 min. The culture of recipient cells was diluted 10, 100, and 1,000 times in LB broth before it was mixed with the donor to obtain several initial recipient concentrations. After centrifugation the supernatant was discarded, and the pellet was resuspended in 50 μl of LB broth and spotted onto a sterile filter disk (0.22-μm pore size; Millipore) on a LB plate. After 60 or 140 min of incubation at 37°C, the filter was resuspended in 2 ml of saline by vigorous agitation on a Vortex mixer; the agitation also interrupted the mating. Donor, recipient, and transconjugant cells were enumerated by serial dilution and plating on the appropriate media (Fig. 1) by the method of Miles and Misra (19). Drops of 20 μl from the appropriate dilutions were spotted on the agar and allowed to dry before incubation at 37°C. Three drops per dilution were spotted, and four dilutions per petri dish were analyzed. For the lowest dilutions (undiluted, 10^{-1} dilution) classical spread plating was also applied; the two methods resulted in similar cell counts. As controls, donor



and recipient strains were incubated separately on filters, under the same conditions as the mating, and plated on the selection plates. When 50-μl samples of each undiluted suspension of parental cells were spread together on the same selective plate, no transconjugants were found. Plasmids in the transconjugants were extracted by a modification of the method of Kado and Liu (9, 31) and visualized by gel electrophoresis.

Model description. Since all matings were performed for 60 and 140 min, we assume that there was no appreciable growth or death of cells or plasmid loss on the filter during the incubation. These assumptions were satisfactory, since the numbers of donors and recipients did not change during the incubation. The assumption of negligible growth is verified further below. In this paper the terms donor and recipient will be used with respect to the Tra^- plasmid being retromobilized by the Tra^+ plasmid.

(i) Model 1: one-step model. The first model describes what would happen if retromobilization occurs in a single step in a biparental mating (Fig. 1a). Mass action leads to the following differential equations

$$dN/dt = k_{12} \times R \times D \quad (1)$$

$$dR/dt = -k_{12} \times R \times D \quad (2)$$

$$dD'/dt = k_1 \times R \times D \quad (3)$$

$$dD/dt = -k_1 \times R \times D \quad (4)$$

where t is the time (in hours); D is the population of donors of the Tra^- plasmid (in CFU per milliliter); R is the recipient population containing only the Tra^+ plasmid (in CFU per milliliter); D' is the population of donor transconjugants (in CFU per milliliter), that is, donors containing both the Tra^+ and Tra^- plasmids; and N is the population of retrotransconjugants (in CFU per milliliter), that is, recipients containing both the Tra^+ and Tra^- plasmids. The conjugal transfer rate constants (per hour per CFU per milliliter) are k_{12}, representing the one-step retromobilization of the Tra^- plasmid into the recipient (R), and k_1, representing the transfer of the Tra^+ plasmid to the donor cell (D).

The model ignores matings between D' and R, between N and R, and between N and D. Experimental data given below indicate that the retrotransconjugant population size (N) is much smaller than the recipient population size (R) after 1 to 3 h, and so R can be assumed essentially constant; thus, equation 2 can be ignored. Further justification for these assumptions is given below.

The analytical solution for D and N, with boundary conditions $N = 0$ and $D = D_0$ at $t = 0$, is

$$D = D_0 \times (e^{-k_1 \times R \times t}) \quad (5)$$

$$D' = D_0 \times (1 - e^{-k_1 \times R \times t}) \quad (6)$$

$$N = (k_{12}/k_1) \times D_0 \times (1 - e^{-k_1 \times R \times t}) \quad (7)$$

(ii) Model 2: two-step model. The second model incorporates, in a first stage, the formation of donor transconjugants (D') and, in a second stage, the production of retrotransconjugants (N) as a result of encounters between D' and R (Fig. 1b).

FIG. 2. Number of retrotransconjugants (N) obtained after 2.33-h (a) and 1-h (b, c) matings plotted as functions of the number of recipients (R). The curve representing simulation 1 is obtained by linear regression of the data using the simplified model 1. The curve of simulation 2 is a plot of the simplified equation with the best-fitting k values.

The differential equations are as follows

$$dD'/dt = k_1 \times R \times D \quad (8)$$

$$dN/dt = k_2 \times R \times D' \quad (9)$$

$$dD/dt = -k_1 \times R \times D \quad (10)$$

$$dR/dt = -k_2 \times R \times D' \quad (11)$$

An additional parameter is k_2, which is the conjugal transfer rate constant for the mobilization of the Tra^- plasmid from D' to the recipient cell R. Although equations 8 and 10 are the same as equations 3 and 4 of model 1, the retrotransconjugants (N) are assumed to be formed in two steps (equations 8 and 9). Again, as the number of retrotransconjugants is small compared with R, R can be assumed to be constant and equation 11 can be ignored. For the same reason, matings between retrotransconjugant cells (N) and recipient cells (R) and between N and donors (D) are not considered.

Integration of the above equations results in

$$D = D_0 \times e^{-k_1 \times R \times t} \quad (12)$$

$$D' = D_0 \times (1 - e^{-k_1 \times R \times t}) \quad (13)$$

$$N = k_2 \times D_0 \times [R \times t - (1 - e^{-k_1 \times R \times t})/k_1] \quad (14)$$

for boundary conditions $N = 0$, $D' = 0$, and $D = D_0$ at $t = 0$.

(iii) Reduction of models 1 and 2. When the number of D' formed during time t is negligible compared to the number of D, concentrations of both R and D can be assumed constant and equations 7 and 14 of model 1 and 2, respectively, can be reduced to

$$\text{Model 1: } N = k_{12} \times R \times D \times t \quad (15)$$

$$\text{Model 2: } N = k_1 \times k_2 \times R^2 \times D \times t^2/2 \quad (16)$$

As shown in the Appendix, these equations are also obtained when equations 7 and 14 are simplified by Taylor series expansion of the exponential functions in the equations. Validation of the assumptions under the experimental conditions used in this study is given below.

RESULTS

Differentiation between the two models. Discrimination between the two models can be based on the dependence of N on t or R; this is different for the two models. Since growth cannot be neglected when long mating periods are used, matings were only performed with different initial recipient population sizes (R) for short time periods and the number of retrotransconjugants per milliliter (N) was determined. The results of three independent experiments are shown in Table 1. In the first set of matings (set 1), both the donor transconjugants (D') and the retrotransconjugants (N) were determined to calculate k_1 (values for k_1 are identical for models 1 and 2), k_{12}, and $k_1 \times k_2$. This first experiment was repeated twice (without determination of D') with a 60-min mating time instead of 140 min (Table 1, sets 2 and 3) to reduce further any influence of cell growth, effect of decreasing D populations, and impact of matings with N as donors. In the reduced equation of the one-step model, the N is a linear function of R (equation 15). The reduced version of the two-step model, on the contrary, reveals a quadratic relation between N and R (equation 16).

To see whether the relationship between N and R was linear, N was regressed against R. There was a very good correlation: correlation coefficients (r) of the three sets of experiments were 0.999, 0.999, and 0.974. When N was regressed against R^2, a lower correlation was found. This indicates that N is a linear function of R, which is in accordance with the one-step model (simplified equation, equation 15). Since k_{12}, D, and t are assumed to be constant during the time period, k_{12} was calculated from the regression result $k_{12} \times D \times t$. The k_{12} values of the three sets were calculated as 6.6×10^{-13}, 1.7×10^{-13}, and 2.5×10^{-14} h^{-1} $(CFU/ml)^{-1}$. Log-log plots show that the slope of the N-R curve from the simplified model 2 is twice the slope of the curve from model 1 (equations 15 and 16). Our data fit model 1 well but do not fit the assumptions in model 2 (Fig. 2).

TABLE 1. Numbers of recipients (R), donors (D), donor transconjugants (D'), and retrotransconjugants (N) after 2.33- and 1-h matings[a]

Data set (h)	No. of CFU/ml			
	R	D	D'	N
1 (2.33)	6.9×10^8	3.9×10^8	4.3×10^7	3.9×10^5
	8.0×10^7	4.5×10^8	6.0×10^6	4.6×10^4
	8.3×10^6	2.7×10^8	8.5×10^5	2.7×10^3
2 (1)	3.2×10^8	2.7×10^8	ND[b]	1.7×10^4
	4.0×10^7	4.0×10^8	ND	2.0×10^3
	3.5×10^6	2.9×10^8	ND	0.9×10^2
	5.0×10^5	2.8×10^8	ND	2.2×10^1
3 (1)	6.0×10^8	6.0×10^8	ND	0.9×10^4
	5.5×10^7	5.5×10^8	ND	2.8×10^3
	9.7×10^6	6.3×10^8	ND	1.2×10^2
	9.8×10^5	4.7×10^8	ND	1.0×10^1

[a] Strains: R, LE392(RP4); D, CSH52(pSUP202); D', CSH52(pSUP202)(RP4); N, LE392(RP4)(pSUP202).
[b] ND, not determined.

Another approach to determine the adequacy of a model is to look for the independence of parameter estimates from the variables in the experimental regime. The different k values can be calculated according to the following equations:

$$k_1 = 1/(R \times t) \ln[D_0/(D_0 - D')] \quad (17)$$

Equation 17 is derived from equation 13.

$$k_{12} = k_1 \times N/D \times (1 - e^{-k_1 \times R \times t}) \quad (18)$$

Equation 18 is derived from the full model 1 equation 7.

$$k_{12} = N/(R \times D \times t) \quad (19)$$

Equation 19 is derived from the simplified model 1 equation 15.

$$k_2 = N/\{D_0 \times [R \times t - (1 - e^{-k_1 \times R \times t})/k_1]\} \quad (20)$$

Equation 20 is derived from the full model 2 equation 14.

$$k_2 = 2 \times N/(k_1 \times R^2 \times D \times t^2) \quad (21)$$

$$k_1 \times k_2 = 2 \times N/(R^2 \times D \times t^2) \quad (22)$$

Equations 21 and 22 are derived from the simplified model 2 equation 16.

The data in Table 2 confirm the hypothesis that retromobilization occurs as a one-step process: model 1 fits very well with the experimental data, since the values of k_{12} seem to be independent of the number of recipient cells (R). In model 2, however, the value of the overall parameter $k_1 \times k_2$ increases with decreasing R, indicating that this model is not adequate: the model inadequacy is compensated for by parameter variation.

TABLE 2. Values of k calculated from the data of Table 1 with equations 17 through 22[a]

Data set	Model 1				Model 2				
	Simplified		Full		Simplified		Full		
	k_1	k_{12}	k_1	k_{12}	k_1	$k_1 \times k_2$	k_1	k_2	$k_1 \times k_2$
1	0.73×10^{-10}	6.21×10^{-13}	0.73×10^{-10}	6.58×10^{-13}	0.73×10^{-10}	7.72×10^{-22}	0.73×10^{-10}	1.11×10^{-11}	8.06×10^{-22}
	0.72×10^{-10}	5.48×10^{-13}	0.72×10^{-10}	5.51×10^{-13}	0.72×10^{-10}	5.87×10^{-21}	0.72×10^{-10}	8.20×10^{-11}	5.90×10^{-21}
	1.63×10^{-10}	5.16×10^{-13}	1.63×10^{-10}	5.17×10^{-13}	1.63×10^{-10}	5.33×10^{-20}	1.63×10^{-10}	3.28×10^{-10}	5.35×10^{-20}
2	—[b]	1.97×10^{-13}	—	—	—	1.23×10^{-21}	—	—	—
	—	1.25×10^{-13}	—	—	—	6.25×10^{-21}	—	—	—
	—	0.88×10^{-13}	—	—	—	5.07×10^{-20}	—	—	—
	—	1.57×10^{-13}	—	—	—	6.29×10^{-19}	—	—	—
3	—	2.39×10^{-14}	—	—	—	7.96×10^{-23}	—	—	—
	—	9.26×10^{-14}	—	—	—	3.37×10^{-21}	—	—	—
	—	1.96×10^{-14}	—	—	—	4.05×10^{-21}	—	—	—
	—	2.17×10^{-14}	—	—	—	4.43×10^{-20}	—	—	—

[a] k_1, k_2, and k_{12} values are given per hour per CFU per milliliter. $k_1 \times k_2$ values are given per hour squared per (CFU per milliliter) squared.
[b] —, not possible to calculate.

Cross-validation (14) of the one-step model was confirmed by using one data set to identify the model and verifying the outcome of the identified model with other experimental data. The k values of the mating with the lowest number of R from set 2 of Table 2 were used to calculate the number of N for the other matings using the respective number of R and D (Table 1, set 2). The experimental data approach very well the number of N predicted by model 1 and not at all the number predicted by model 2 (Table 3). The same conclusion could be drawn when other identification and validation sets were used.

Possible contribution of model 2 to model 1. Model 1 assumes that matings between D' and R, occurring in a second step, do not contribute to the formation of retrotransconjugants. The good fit of model 1 seems to indicate that retrotransfer is a one-step conjugation process but does not exclude a certain contribution of the two-step transfer process as simulated by model 2. Therefore, we investigated what contribution of model 2 is required before differences between experimental data and simulated values can be detected. Simulations were performed with a model combining models 1 and 2, in which f is defined as the fraction of the overall model corresponding with model 2; i.e., an f of 0% would indicate simulation with 100% model 1, and an f of 100% would indicate simulation with 100% model 2. The kinetic parameters were calculated from data set 1 (Tables 1 and 2) for $R = 8.3 \times 10^6$ CFU/ml and subsequently used in the model to predict the number of retrotransconjugants N at higher R concentrations (e.g., $R = 6.9 \times 10^8$ CFU/ml). The percentages of error on estimated values relative to the experimental value of N with different values of f were as follows: for $f = 0\%$, -26.2% error (negative value indicates underestimation); for $f = 1\%$, 37.2% error (positive value indicates overestimation); for $f = 2.5\%$, 135% error; for $f = 5\%$, 290% error. The value of N obtained by simulation using 95% of model 1 and 5% of model 2 deviates already by a factor of 4 from the experimental value of N ($N = 3.9 \times 10^5$ CFU/ml). This means that the contribution of a two-step process could not be more than 5% and can thus be neglected.

TABLE 3. Cross-validation of the one-step model (model 1) and the two-step model (model 2)[a]

Data set	N (CFU/ml)		
	Experimental data[a]	Model 1	Model 2
Identification	2.2×10^1	2.2×10^1	2.2×10^1
Cross-validation	0.9×10^2	1.6×10^2	1.2×10^3
	2.0×10^3	2.5×10^3	2.0×10^5
	1.7×10^4	1.3×10^4	8.7×10^6

[a] Experimental data are from data set 2 of Table 1. Both the experimental and simulated data represent the number of N after 1 h.

Validation of the assumptions made to simplify the models. The consequences of the assumptions used to simplify both models were explored by using computer simulations. If not stated otherwise, the reference k, R_0, and D_0 values used in the simulations are based on the following calculated values derived from the experimental data (Tables 1 and 2): $k_{12} = 5.0 \times 10^{-13}$, $k_1 = 1.0 \times 10^{-10}$, $k_2 = 1.0 \times 10^{-11}$ h^{-1} (CFU/ml)$^{-1}$; $R_0 = 1.0 \times 10^8$ CFU/ml; $D_0 = 5.0 \times 10^8$ CFU/ml.

(i) Neglect of cell growth. By adding a growth term ($\mu \times Z$) ($Z = R, D, D'$, or N; μ is the growth rate per hour, which in this case is assumed to be equal for all cell lines) to all differential equations (equations 1 through 4 and 8 through 11), the effect of cell growth on the predicted number of transconjugants and retrotransconjugants was investigated. When $\mu = 0.4$ h^{-1}, the number of retrotransconjugants (N) after 1 h, simulated by model 1 including growth, doubled compared with that in the situation of no growth. This indicates that for higher μ values the assumptions of no growth no longer obtain. Experimental data, however, showed that no detectable growth occurs during the mating period. Even after 24 h, cells on the filter only increased by a factor of 10, corresponding with $\mu = 0.1$ h^{-1}. Therefore growth can be neglected in our models.

(ii) Neglect of matings between retrotransconjugants (N) and recipients (R) or donors (D). The influence of adding a term $k_3 \times R \times N$ to the differential equations 1, 2, 9, and 11 and a term $k_4 \times D \times N$ to the equations 3, 4, 8, and 10 was investigated. No significant effect of retrotransconjugant-recipient matings on the total number of retrotransconjugants could be observed for k_3 values ranging from 10^{-12} to

TABLE 4. Number of donors D obtained by computer simulation with the full model 1 after 1- and 3-h matings[a]

Time (h)	D (CFU/ml) for R (CFU/ml) of:				
	1×10^8	2×10^8	4×10^8	8×10^8	1.6×10^9
1	4.95×10^8	4.90×10^8	4.80×10^8	4.62×10^8	4.26×10^8
3	4.85×10^8	4.71×10^8	4.43×10^8	3.93×10^8	3.03×10^8

[a] The initial value of D was 5.0×10^8 CFU/ml; $k_1 = 10^{-10}$ h^{-1} (CFU/ml)$^{-1}$.

10^{-8} h^{-1} (CFU/ml)$^{-1}$. For $k_3 = 10^{-8}$ h^{-1}(CFU/ml)$^{-1}$, N predicted by the extended model 1 increased by only 4%. Also the number of D' was not affected by retrotransconjugant-donor matings for k_4 values in the same range.

(iii) Neglect of the decrease of R and D during the time interval. R was assumed to be constant during the conjugation period; this drastically simplified our models. The reduced models were obtained by assuming D to be constant too. The results shown in Table 1 clearly demonstrate that the fraction of recipients (R) transformed into retrotransconjugants (N) during 2.3 or 1 h is indeed very small ($<10^{-3}$) and can be neglected; therefore the number of R can be assumed to be constant.

The number of D cells decreases more rapidly because of rapid formation of donor transconjugants (D'). Only for R values of 6.0×10^8 CFU/ml and higher, changes in D can no longer be neglected (Table 4), and the simplified model is no longer valid.

The same observation can be made by comparing the values of k obtained by the simplified and full models. The k_{12} values calculated with the full model 1 are very similar to those obtained with the simplified model 1 (Table 2), indicating that the assumptions made to simplify the model are valid. Also, in model 2, the k_2 values calculated with the simple model agree very well with those obtained with the full model. The very small underestimation of the k values after simplification of both models becomes more pronounced when the number of recipient cells increases: for k_{12} a deviation of 5.6% is observed and for k_2 a deviation of 4.2% is observed in the mating with the highest number of R (6.9×10^8 CFU/ml). This is in agreement with the conclusions from Table 4.

The same conclusion can also be drawn by applying the Taylor series expansion (see Appendix); reducing the equations is allowed if the neglected terms contribute for only 1% or less, which is obtained when $R < 0.02/k_1$.

Finally the number of retrotransconjugants (N) was simulated with the full and simplified models. The effects of reduction of models 1 and 2 (percentages of deviation of N relative to values obtained with the full models) are as follows: at 1 h (mating time), 0.4% for both models; at 3 h, 1.5% for model 1 and 0.9% for model 2. (These results were calculated from the following values: $R = 1 \times 10^8$ CFU/ml, $D = 5 \times 10^8$ CFU/ml, $k_1 = 10^{-10}$ h^{-1} [CFU/ml]$^{-1}$, $k_2 = 10^{-11}$ [CFU/ml]$^{-1}$, $k_{12} = 5 \times 10^{-13}$ [CFU/ml]$^{-1}$.) The simplified models are as valid as the full models under these experimental conditions.

DISCUSSION

We demonstrated that both the transfer and retrotransfer rate constants (k_1 and k_{12}) are independent of the donor/recipient ratio. Levin et al. (13) also observed that the transfer rate constant for a classical direct plasmid transfer is relatively independent of the relative frequency of donors and recipients. These authors believe that the mass action model, with its implicit assumption of a dimensionless habitat, could not serve for modeling plasmid transfer in patchy habitats. However, the mass action model has been successfully used for prediction of plasmid transfer in solid habitats like soil (2, 10). The usefulness of the mass action model for predicting plasmid transfer dynamics on surfaces was investigated further by use of a surface slide system (24). The model did simulate the plasmid transfer dynamics when high inoculum concentrations were used. When the parental strains were inoculated at lower concentrations, the number of transconjugants determined after 30 h (steady state) was not comparable to the numbers obtained in matings in liquid cultures. Simonsen therefore concluded that the mass action model, which obtains for matings in liquids, could not be used for matings on surfaces. Nevertheless, it is possible that the model can be used if surface cultures are initiated with a high cell density ($>10^6$ cells cm^{-2}) at a donor/recipient ratio of 1:1 (26). In our mating procedure, the surface of the filter covered by the 50-μl drop is ca. 0.5 cm^2. Even at the lowest concentration of R, a density of ca. 2×10^6 CFU cm^{-2} of recipients is reached. Moreover, the incubation time was only 60 or 140 min and therefore the results cannot be compared with those of a 30-h mating. Since the relationship between N and R as described in our model 1 and the relationship between D' and R fit our data very well, we believe that the mass action model can also be used in filter matings under the conditions applied in this study. The variation in the values of the retrotransfer rate constant k_{12} obtained in the three sets of experiments could be due to small differences in mating conditions. The effect of energy availability on the conjugative-transfer kinetics of plasmid RP4 was recently demonstrated (15). A decrease of substrate availability significantly decreased the transconjugant formation rate. The transfer rate constant also varied with the amount of time the parental cells were incubated before being harvested for a transfer experiment (15). Our variations in k_{12} could also be explained by the latter phenomenon, since small variations in culture age are unavoidable.

The complete mass action model includes several processes (13). This study demonstrates that some of these processes can be neglected under certain mating conditions. By limiting the conjugation time period to 1 h or a maximum of 2.3 h, the models could be reduced to simple equations. During this short time period, cell growth on the filter can be neglected, and the numbers of recipients and (for recipient numbers that are not too high) also donors can be assumed to be constant. Also, matings between retrotransconjugants (N) and either recipients or donors are negligible because of the low number of N formed within this time interval. Hence, the relationship between N and R is reduced to a linear function in the one-step model and to a quadratic function in the two-step model, allowing easy distinction between the models.

Models were selected by using three approaches (14). First, the relationship between N and R was shown to be linear and not quadratic, indicating that model 1 fits the data. The study of parameter invariance was another approach; since the k_{12} values estimated by the one-step model were independent of the number of recipients, whereas those of the two-step model were not, the one-step model was selected. Finally, an attractive way of comparing two different models is to evaluate their performances when applied to a data set to which neither of them has been adjusted. Such a procedure is known as cross-validation (14). This third

procedure indicated that only the one-step model fits our data. So the results of these three approaches confirm the hypothesis that retrotransfer of plasmids is the result of only one encounter between two cells and that the formation of retrotransconjugants is first order with respect to the number of recipient cells (R), which simultaneously act as donors of the conjugative plasmids. Hence, retrotransfer appears to be a one-step process of bidirectional DNA transfer, which is different from a triparental mating during which at least two encounters are needed. Retrotransfer is thus a specific phenomenon that could have interesting implications for gene exchange in nature, since gene mobilization through this mechanism requires only two mating partners and one collision and could therefore become more important than triparental matings in natural (especially heterogeneous) habitats where the probability of cell encounters is lower than in dense cultures. Moreover, retromobilization allows the host to capture genes from species in which the retromobilizing plasmid cannot be stably established (20). The molecular mechanism of retrotransfer is not yet fully understood, yet the findings described in this paper strengthen the hypothesis that retrotransfer is a conjugation mechanism that could be separated from direct transfer and mobilization (17). Further study is needed to elucidate the phenomenon completely.

The mathematical model developed in this study could be expanded to study retrotransfer in soil habitats. It could be a useful tool for assessing the probability of uptake of foreign DNA by autochtonous microorganisms harboring conjugative, retromobilizing plasmids when allochtonous bacteria are introduced into the soil environment. The model developed by Knudsen et al. (19) could, according to the authors, be used as a first step in predicting mobilization of nonconjugative plasmids. The results described in this study show that these models could also be used for retromobilization of nonconjugative plasmids.

APPENDIX

Taylor series expansions of exponential functions are as follows: $e^x = 1 + x + x^2/2! + x^3/3! + \ldots$ and $e^{-k_1 \times R \times t} = 1 - k_1 \times R \times t + {k_1}^2 \times R^2 \times t^2/2 - \ldots$.

Model 1. In model 1, $N = (k_{12}/k_1) \times D_0(1 - e^{-k_1 \times R \times t}) = (k_{12}/k_1) \times D_0(1 - 1 + k_1 \times R \times t - {k_1}^2 \times R^2 \times t^2/2 + \ldots)$, $N = k_{12} \times D_0(R \times t - k_1 \times R^2 \times t^2/2 + \ldots)$, and if $R \leq 1/(50 \times k_1)$, then the third term of the Taylor series expansion formula is 100 times smaller than the second term (for $t = 1$ h) and can be neglected. Therefore, N can be reduced to $k_{12} \times R \times D \times t$ for $D = D_0$. If $k_1 = 10^{-10}$, R should be 2×10^8 maximum, which is almost the case in our matings with the highest R concentration and certainly in all other matings.

Model 2. In model 2, $N = k_2 \times D_0[R \times t - (1 - e^{-k_1 \times R \times t})/k_1] = k_2 \times D_0[R \times t - (1 - 1 + k_1 \times R \times t - {k_1}^2 \times R^2 \times t^2/2 + \ldots)/k_1]$ (in this case the fourth term of the Taylor series expansion formula can be neglected for the reasons stated above) $= k_2 \times D_0(R \times t - R \times t + k_1 \times R^2 \times t^2/2)$, $N = k_1 \times k_2 \times D_0 \times R^2 \times t^2/2$, and $N = k_1 \times k_2 \times D \times R^2 \times t^2/2$ for $D = D_0$.

ACKNOWLEDGMENTS

E. Top is Research Assistant of the Belgian National Fund for Scientific Research. This work was partially supported by the European Community BAP program BAP 0367-B and the European Community BRIDGE program BIOT-CT91-0284.

We are grateful to V. Vandepitte for his help with the scientific graph system program Sigma Plot. We thank N. McClure for providing the strain LE392 Rif^r. We express our gratitude to J. C. Fry for improving the manuscript. We also thank the reviewers of the manuscript for their useful suggestions.

REFERENCES

1. **Buchanan-Wollaston, V., J. E. Passiatore, and F. Cannon.** 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature (London) **328:**172–175.
2. **Clewlow, L. J., N. Cresswell, and E. M. H. Wellington.** 1990. Mathematical model of plasmid transfer between strains of streptomycetes in soil microcosms. Appl. Environ. Microbiol. **56:**3139–3145.
3. **Datta, N., R. W. Hedges, E. J. Shaw, R. B. Sykes, and M. H. Richmond.** 1971. Properties of an R factor from *Pseudomonas aeroginosa*. J. Bacteriol. **108:**1244–1249.
4. **Dennison, S., and S. Baumberg.** 1975. Conjugational behaviour of N plasmids in *Escherichia coli* K12. Mol. Gen. Genet. **138:**323–331.
5. **Freter, R., R. R. Freter, and H. Brickner.** 1983. Experimental and mathematical models of *Escherichia coli* plasmid transfer in vivo and in vitro. Infect. Immun. **39:**60–84.
6. **Gormley, E. P., and J. Davies.** 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Streptomyces lividans* and *Mycobacterium smegmatis*. J. Bacteriol. **173:**6705–6708.
7. **Guiney, D. G., P. Hasegawa, and C. E. Davis.** 1984. Plasmid transfer from *Escherichia coli* to *Bacteroides fragilis*. Differential expression of antibiotic resistance genes. Proc. Natl. Acad. Sci. USA **81:**7203–7206.
8. **Heinemann, J. A., and G. Sprague.** 1989. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature (London) **340:**205–209.
9. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
10. **Knudsen, G. R., M. V. Walter, L. A. Porteous, V. J. Prince, J. L. Armstrong, and R. J. Seidler.** 1988. Predictive model of conjugative plasmid transfer in the rhizosphere and phyllosphere. Appl. Environ. Microbiol. **54:**343–347.
11. **Kreps, S., F. Ferino, C. Mosrin, J. Gerits, M. Mergeay, and P. Thuriaux.** 1990. Conjugative transfer and autonomous replication of a promiscuous IncQ plasmid in the cyanobacterium *Synechocystis* PCC 6803. Mol. Gen. Genet. **221:**129–133.
12. **Levin, B. R., and F. M. Stewart.** 1980. The population biology of bacterial plasmids: a priori conditions for the existence of mobilizable nonconjugative factors. Genetics **94:**425–443.
13. **Levin, B. R., F. M. Stewart, and V. A. Rice.** 1979. The kinetics of conjugative plasmid transmission: fit of a simple mass action model. Plasmid **2:**247–260.
14. **Ljung, L.** 1987. Model structure selection and model validation, p. 408–431. *In* L. Ljung (ed.), System identification—theory for the user. Prentice-Hall, Inc., Englewood Cliffs, N.J.
15. **MacDonald, J. A., B. F. Smets, and B. E. Rittmann.** 1992. The effects of energy availability on the conjugative-transfer kinetics of plasmid RP4. Water Res. **26:**461–468.
16. **Mazodier, P., R. Petter, and C. Thompson.** 1989. Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. J. Bacteriol. **171:**3583–3585.
17. **Mergeay, M., P. Lejeune, A. Sadouk, J. Gerits, and L. Fabry.** 1987. Shuttle transfer (or retrotransfer) of chromosomal markers mediated by plasmid pULB113. Mol. Gen. Genet. **209:**61–70.
18. **Mergeay, M., D. Springael, and E. Top.** 1990. Gene transfer in polluted soils, p. 152–171. *In* J. C. Fry and M. C. Day (ed.), Bacterial genetics in natural environments. Chapman & Hall, London.
19. **Miles, A. A., and S. S. Misra.** 1938. The estimation of the bactericidal power of blood. Hygiene **38:**732–740.
20. **Powell, B., M. Mergeay, and N. Christofi.** 1989. Transfer of broad host range plasmids to sulphate-reducing bacteria. FEMS Lett. **59:**269–274.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Schäfer, A., J. Kalinowski, R. Simon, A.-H. Feldhaus, and A. Pühler.** 1990. High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive cory-

neform bacteria. J. Bacteriol. **172:**1663–1666.
23. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilisation system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
24. **Simonsen, L.** 1990. Plasmid transfer dynamics on surfaces. J. Gen. Microbiol. **136:**1001–1007.
25. **Simonsen, L.** 1991. The existence conditions for bacterial plasmids: theory and reality. Microb. Ecol. **22:**187–205.
26. **Simonsen, L., D. M. Gordon, F. M. Stewart, and B. R. Levin.** 1990. Estimating the rate of plasmid transfer: an end point method. J. Gen. Microbiol. **136:**2319–2325.
27. **Springael, D.** 1991. Genetics of PCB-degrading bacteria: identification of a new catabolic transposon Tn*4371*. Ph.D. thesis. University of Brussels, Brussels, Belgium.
28. **Thiry, G. M., M. Mergeay, and M. Faelen.** 1984. Back-mobilization of Tra^- Mob^+ plasmids mediated by various IncM, IncN and IncP1 plasmids. Arch. Int. Physiol. Biochim. **92:**B64–B65.
29. **Thomas, C. M., and C. A. Smith.** 1987. Incompatibility group P plasmids: genetics, evolution, and use in genetic manipulation. Annu. Rev. Microbiol. **41:**77–101.
30. **Toda, K., P.-X. Yao, and H. Ohtake.** 1990. Application of multi-stage continuous culture for estimating net rate of plasmid transfer. J. Gen. Appl. Microbiol. **36:**279–282.
31. **Top, E., M. Mergeay, D. Springael, and W. Verstraete.** 1990. Gene escape model: transfer of heavy metal resistance genes from *Escherichia coli* to *Alcaligenes eutrophus* on agar plates and in soil samples. Appl. Environ. Microbiol. **56:**2471–2479.
32. **Trieu-Cuot, P., C. Carlier, M. Martin, and P. Courvalin.** 1987. Plasmid transfer by conjugation from *Escherichia coli* to gram-positive bacteria. FEMS Microbiol. Lett. **48:**289–294.
33. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *E. coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
34. **Wolk, C. P., A. Vonshak, P. Kehoe, and J. Elhai.** 1984. Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. Proc. Natl. Acad. Sci. USA **81:**1561–1565.