

Predictive Model of Conjugative Plasmid Transfer in the Rhizosphere and Phyllosphere

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A computer simulation model was used to predict the dynamics of survival and conjugation of *Pseudomonas cepacia* (carrying the transmissible recombinant plasmid R388:Tn1721) with a nonrecombinant recipient strain in simple rhizosphere and phyllosphere microcosms. Plasmid transfer rates were derived for a mass action model, and donor and recipient survival were modeled as exponential growth and decay processes or both. Rate parameters were derived from laboratory studies in which donor and recipient strains were incubated in test tubes with a peat-vermiculite solution or on excised radish or bean leaves in petri dishes. The model predicted donor, recipient, and transconjugant populations in hourly time steps. It was tested in a microcosm planted with radish seeds and inoculated with donor and recipient strains and on leaf surfaces of radish and bean plants also growing in microcosms. Bacteria were periodically enumerated on selective media over 7 to 14 days. When donor and recipient populations were 10^6 to 10^8 CFU/g (wet weight) of plant or soil, transconjugant populations of about 10^1 to 10^4 were observed after 1 day. An initial rapid increase and a subsequent decline in numbers of transconjugants in the rhizosphere and on leaf surfaces were correctly predicted.

The ability to predict the survival and dissemination of recombinant gene sequences in nature is essential in the assessment of risk due to released genetically engineered bacteria. Conjugative plasmids, with their ability to undergo transfer and to mobilize other plasmids, probably represent the greatest potential for gene transfer and are thus useful as a worst-case model system. Because research with recombinant microorganisms is currently permitted only in contained settings, results from experiments in relatively simple systems (i.e., in vitro or in microcosms) must be extrapolated to complex environmental situations. It would be valuable to know to what extent results from the simpler systems can be used to predict events in more complex systems.

Levin et al. (3) used a mass action model to describe the kinetics of conjugative plasmid transmission (i.e., production of transconjugants is directly proportional to the product of donor and recipient concentrations) in log-phase and stationary-phase cultures of *Escherichia coli*. Freter et al. (1a) expanded the model to evaluate *E. coli* plasmid transfer in human and animal guts. It is not known whether such models can predict transconjugation events in the phyllosphere (aerial plant surfaces) and rhizosphere (plant root environment). Since microbial survival and interactions on plants and in soil may be affected by a fluctuating microenvironment, a model to predict the dynamics of plasmid transfer under these conditions must consider host, recipient, and transconjugant population dynamics. A computer simulation approach allows survival and/or plasmid transfer rates to vary over time in response to a changing environment. The objective of this research effort was to develop a computer simulation model, by using easily obtained laboratory data, to make predictions about bacterial survival and

plasmid transfer in a more complex system. We describe the derivation and structure of the model, experiments to obtain the parameters used in it, and independent validation experiments in rhizosphere and phyllosphere microcosms.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two strains of *Pseudomonas cepacia* served as the donor and recipient. The donor was *P. cepacia* PCO1215(R388::Tn1721), a proline auxotroph carrying the transmissible recombinant plasmid R388::Tn1721, which carries genes encoding resistance to tetracycline, trimethoprim, and sulfonamide. The donor strain is sensitive to nalidixic acid. Donor cells were enumerated by using Luria-Bertani (LB [4]) broth or agar, amended with 50 μ g of trimethoprim per ml. The recipient strain was *P. cepacia* PCO1200, a prototroph resistant to nalidixic acid and sensitive to trimethoprim. Recipient cells were enumerated by using M9 (6) broth or agar amended with 500 μ g of nalidixic acid per ml. Transconjugants were selected by using M9 medium containing trimethoprim and nalidixic acid. Bacteria were grown to late log phase in LB broth with appropriate antibiotics added and then washed twice in phosphate buffer (1.2 mM KH_2PO_4 [pH 7.2]) before use. When bacteria were added to nonsterile soil or leaf surfaces, cycloheximide (100 μ g/ml) was added to the isolation media.

Model description. The computer simulation model incorporates (i) donor, recipient, and transconjugant survival dynamics and (ii) conjugative plasmid transfer dynamics, to predict populations of donors, recipients, and transconjugants in hourly time steps. Bacterial survival was modeled as a simple exponential growth-decay process. Thus, the population at time t (in hours), $N_t = N_{t-1} \times 10^\beta$, where β is a rate parameter. Plasmid transfer (i.e., formation of transconjugants from recipients) was modeled by using the mass action model of Levin et al. (3). It was assumed that transconjugants would act as plasmid donors and would

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exhibit similar survival characteristics to those of the donor strain. Model equations were as follows:

$$D_t = D_{t-1} \times 10^{\beta_1}$$

$$R_t = R_{t-1} \times 10^{\beta_2} - \tau[(D_{t-1} + T_{t-1}) \times R_{t-1}]$$

$$T_t = T_{t-1} \times 10^{\beta_1} + \tau[(D_{t-1} + T_{t-1}) \times R_{t-1}]$$

where t is the time (in hours), D_t is the donor population (in CFU per gram), R_t is the recipient population (in CFU per gram), T_t is the transconjugant population (in CFU per gram), β_1 is the rate parameter for donor and transconjugant populations, β_2 is the rate parameter for recipient populations, and τ is the conjugational transfer rate constant (defined below). The model was written in Pascal and implemented on an IBM microcomputer.

Bacterial survival and plasmid transfer rates in PVM. Survival rates for *P. cepacia* strains in a nonsterile peat-vermiculite mixture (PVM) were obtained from experiments described by Walter et al. (8), in which aqueous PVM suspensions in test tubes were inoculated with bacteria (10^5 /ml) and incubated at 30°C. Populations were monitored over a 13-day period, and exponential survival curves were fitted to the data by using linear regression of log-transformed population data.

The rate of plasmid transfer in sterile PVM suspensions was obtained as follows: PVM (5 g per tube) was added to 18 ml of phosphate buffer in each of three screw-cap test tubes, and the tubes were autoclaved for 10 min. Donor and recipient cells (1 ml, 5×10^8 CFU/ml) were added, and the tubes were shaken briefly and then incubated for 20 h at 30°C. Samples were serially diluted and plated onto appropriate media to enumerate donor, recipient, and transconjugant populations. The plasmid transfer rate constant was estimated as $\tau = (\text{new transconjugants per hour}) \times D_{t-1} \times R_{t-1}$, where donor and recipient numbers are means of initial and final population counts.

Bacterial survival and plasmid transfer rates on leaf surfaces. Estimates of donor and recipient survival rates on leaf surfaces were obtained from experiments by Knudsen and Spurr (G. R. Knudsen and H. W. Spurr, Jr., in K. G. Mukerji, ed., *Biocontrol of Plant Diseases*, in press) in which a strain of *P. cepacia* was applied to peanut and tobacco leaves in a controlled-environment chamber, and maintained under varying high (>95%) or low (ca. 60%) relative humidity (RH) for periods up to 96 h. Exponential curves were fitted to population growth (high RH) or decay (low RH) trends. These rates were used to estimate donor and recipient population changes on leaf surfaces.

A plasmid transfer rate for leaf surfaces was estimated by using 3-week-old radish (*Raphanus sativus* cv. Cherry Belle) and bean (*Phaseolus vulgaris humilis* cv. Bush Blue Lake) plants grown indoors in nonsterile PVM. Filter paper disks in 9-cm-diameter plastic petri dishes were wetted with 0.5 ml of phosphate buffer, and one to three excised leaves were placed in each dish. Donor and recipient strains were diluted to concentrations of about 10^6 , 10^7 , 10^8 , and 10^9 CFU/ml, 20 5- μ l drops of donor cell suspension at each of the above concentrations were placed onto leaves in each of six petri dishes, and 20 5- μ l drops of recipient cell suspension at the same concentration were immediately added. Sizes of donor and recipient populations were estimated by placing sample leaves from three petri dishes into plastic bags with 10 ml of phosphate buffer each and blending them for 1 min in a stomacher blender (Tekmar Co., Cincinnati, Ohio). After serial dilution in phosphate buffer, 33- μ l samples were added to 167 μ l of LB containing trimethoprim (for donors), M9

medium containing nalidixic acid (for recipients), or M9 medium containing trimethoprim and nalidixic acid (for transconjugants), in wells of 96-well microtiter plates, 8 wells per dilution. The remaining three petri dishes were sealed with laboratory film to retard evaporation and incubated at 30°C for 20 h, when leaves were again sampled. In addition, a 0.5-ml sample from the blender bag was serially diluted and added to test tubes (five tubes per dilution) containing 4.5 ml of M9 medium containing trimethoprim and nalidixic acid to select for transconjugant cells that might be present in very low numbers. Microtiter plates and tubes were incubated for 48 h at 30°C, and turbid wells and tubes were counted. Estimates of donor, recipient, and transconjugant CFU per leaf were made by comparing counts with most-probable-number tables (5). Mean donor and recipient population levels over the 20-h period were estimated by averaging initial and final counts, and the plasmid transfer rate constant was calculated as above. The experiment was performed three times, and the mean value of τ was used in the model, for conditions of high (>95%) RH. For the model it was assumed that plasmid transfer did not occur ($\tau = 0$) during times when leaf surfaces were dry, as estimated by low (<95%) RH. Linear regression of observed τ values against the product of donor and recipient cell density was performed for the data set, to determine whether τ was independent of cell density. Regression was also performed on the same data set after logarithmic transformation of both variables.

Testing the model in a rhizosphere microcosm. About 90 radish seeds were distributed over the surface of PVM in 6-in. pots. Donor and recipient cell suspensions (15 ml each; 10^9 CFU/ml in phosphate buffer) were poured over the seeds, which were then lightly covered with PVM. Pots were kept in enclosed glass chambers on a 8-h/16-h light/dark cycle; the ambient temperature was about 28 to 30°C (light) and 22°C (dark). Single bulked samples were taken periodically, up to 12 days after planting. On days 0 and 1, samples consisted of about 1 g of PVM and seeds; subsequent samples consisted of about 5 g of plant roots plus associated PVM (rhizosphere). Samples were placed in tubes with Tris buffer (10 mM [pH 7.5]) and agitated for 1 min, and then serial dilutions were made and plated onto selective media. Donor, recipient, and transconjugant populations were enumerated after 48 h of incubation at 30°C. Samples were also plated on LB agar to enumerate total LB-culturable bacteria. The experiment was performed three times. For each experiment, the simulation model was calibrated by using donor and recipient numbers observed at time zero. The model then predicted subsequent donor, recipient, and transconjugant populations.

Testing the model in a phyllosphere microcosm. Donor and recipient strains were suspended in phosphate buffer at about 5×10^8 CFU/ml. Three-week-old radish or bean plants growing in microcosms were sprayed with bacterial suspensions until leaf surfaces were wet. The microcosms, which have been described previously (1), were plastic trays containing 5 cm of PVM and enclosed by plastic bags, inside larger glass chambers. Relative humidity levels were estimated from readings taken in adjacent microcosms at the same time by using an RH sensor and datalogger (Campbell Scientific Inc., Logan, Utah). When the plastic bags were kept closed, RH was greater than or equal to 95% during the 8-h dark period and about 85% during the 16-h light period. When the bags were open, RH was 25 to 30%. Four experiments were performed, each lasting 14 days. In experiment 1, donor and recipient strains were applied to radish

foliage and the plastic bag was kept closed, except during sampling. In experiment 2, strains were applied to radish foliage and the bag was kept open on days 1 to 2, 3 to 5, and 7 to 14 to observe effects of fluctuating RH. Each time the bag was closed, the foliage was misted with sterile water. In experiment 3, strains were applied to bean foliage and the bag was kept closed. In experiment 4, the recipient strain was applied to bean foliage and the donor strain was applied 1 day later. The bag was kept closed for the duration of the experiment. Bulk samples were taken randomly from the plant canopy and consisted of 2 to 6 g of radish or 5 to 12 g of bean foliage. Samples were blended as described above, and serial dilutions were added to microtiter plates with selective media or LB broth. Serial dilutions were also added separately to test tubes containing selective media (0.5-ml sample into 4.5 ml). The level of detection for culturable cells was about 1 CFU/g for test tubes, compared with about 30 CFU/g for microtiter plates. For each experiment, the simulation model was calibrated by using donor and recipient numbers observed at time zero. For each hour of the simulation, RH values were read from a data file, and the model then predicted donor, recipient, and transconjugant populations.

RESULTS

Survival and plasmid transfer rate parameters. Rate parameters obtained from previous reports and in the above experiments are listed in Table 1. Mean values for the plasmid transfer rate constant, τ , were similar in PVM ($\tau = 5.0 \times 10^{-14}$) and on leaf surfaces ($\tau = 5.6 \times 10^{-14}$). Values of τ observed when leaf disks were inoculated with different cell densities ranged from 1.8×10^{-15} to 2.4×10^{-13} . When the value of τ was regressed against the product of donor and recipient average populations on leaf disks, no significant correlation was observed ($r = -0.39$; not significant at the 5% level). However, when $\log \tau$ was regressed against \log (donor \times recipient populations), a significant (5% level; $r = -0.75$) correlation was observed. Thus, the transfer rate constant was higher at the lower cell densities.

Microcosm experiments. In all experiments, transconjugants could be isolated from rhizosphere or phyllosphere microcosms after 1 day. In no case were transconjugants observed at time zero. Generally, the highest numbers of transconjugants were observed 1 to 2 days after microcosms were inoculated with donor and recipient strains.

Figure 1A to C show results of the three rhizosphere microcosm experiments, compared with the simulation model predictions of donor, recipient, and transconjugant populations. Confidence intervals (95%) around sample means are shown. In all three experiments, the model correctly predicted an initial rapid rise (within 1 to 2 days) and subsequent decline in transconjugant populations. In Fig. 1A and C, predictions of transconjugant populations were (with one exception) within 1 log value of experimental observations. Predicted donor and recipient populations also were close to the observed values, although the predicted rates of decline were somewhat faster than those observed. In Fig. 1B, predicted recipient populations were lower than observed populations. This may reflect a deviation from predicted decline rates or an underestimation of the initial population level. As a result, predictions of transconjugant populations also were lower than the observed values. However, transconjugant population trends were predicted correctly.

In the phyllosphere microcosm tests, the model again correctly predicted an initial rapid increase in numbers of transconjugants (Fig. 2A to D). In each of the four tests, the predicted number of transconjugants after 1 day fell within the 95% confidence interval. The effect of changes in RH on predicted bacterial populations is shown by fluctuations in the population curves in Fig. 2A to D. In all cases, the model predicted a reduction in donor and recipient populations over time.

On radish leaves (Fig. 2A and B), populations of donor, recipient, and transconjugant strains declined more rapidly than predicted on days 1 to 5. Predicted donor populations at day 14 were within confidence intervals in both experiments, but predicted recipient populations were above confidence intervals in both experiments. On bean leaves (Figs. 2C and D), predicted populations more closely corresponded to observed populations. We observed that the bean foliage was considerably more dense and filled the microcosms more than radish foliage and that the bean foliage appeared to retain leaf surface wetness for longer periods. On bean leaves, predicted numbers of donors and recipients at day 14 fell within confidence intervals in both experiments, whereas predicted numbers of transconjugants were within the confidence interval in one experiment and slightly below in the other.

In both rhizosphere and phyllosphere experiments, differences between numbers of bacteria observed with selective media and those observed with LB were highly variable.

TABLE 1. Bacterial survival and plasmid transfer rate parameters

| Parameter | <i>P. cepacia</i> strain and microcosm | Parameter value |
|--|--|-----------------------|
| Growth-death rate (β) ^a | PCO1215(R388::Tn1721) (donor) | |
| | Rhizosphere | -0.011 |
| | Phyllosphere (RH > 95%) | 0.005 |
| | Phyllosphere (RH < 95%) | -0.009 |
| | PCO1200 (recipient) | |
| | Rhizosphere | -0.008 |
| | Phyllosphere (RH > 95%) | 0.005 |
| | Phyllosphere (RH < 95%) | -0.009 |
| Plasmid transfer rate constant (τ) ^b | Rhizosphere | 5.0×10^{-14} |
| | Phyllosphere (RH > 95%) | 5.6×10^{-14} |
| | Phyllosphere (RH < 95%) | 0.0 |

^a β , Hourly exponential (base 10) population change rate.

^b Plasmid transfer rate constant, τ , is estimated as follows: $\tau = (\text{new transconjugants per hour}) \times (\text{donors})^{-1} \times (\text{recipients})^{-1}$, where donor and recipient numbers are mean population levels over 20 h in experiments performed with PVM suspensions or excised radish and bean leaves.

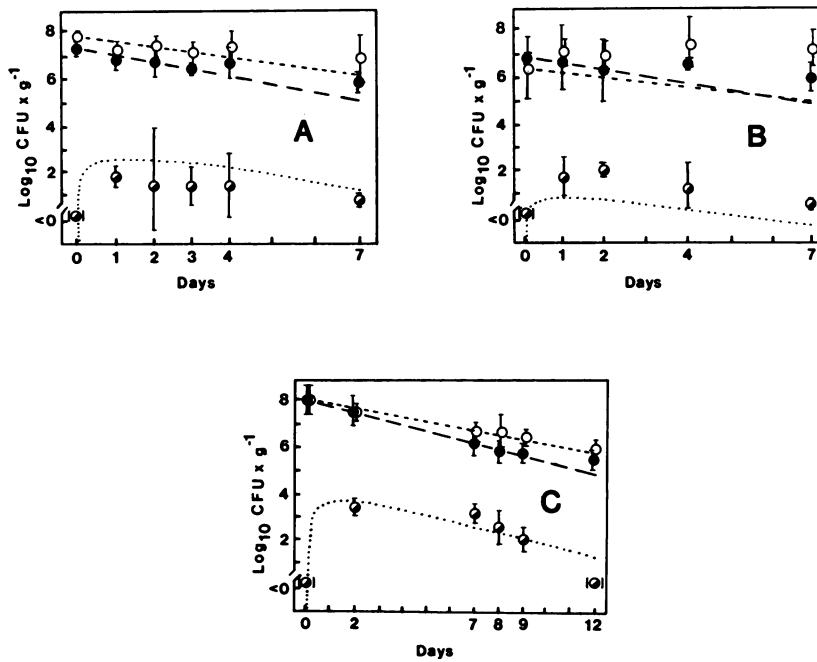


FIG. 1. Observed and predicted *P. cepacia* populations in rhizosphere microcosms in three experiments (A, B, and C). Predictions were made by calibrating the simulation model with observed populations at time zero. Symbols: ●, donor observed; —, donor predicted; ○, recipient observed; - - - - -, recipient predicted; ●, transconjugant observed;, transconjugant predicted. Confidence intervals (95%) around sample means are indicated by vertical bars. Points shown in brackets on days 0 and 12 represent populations below the detection threshold.

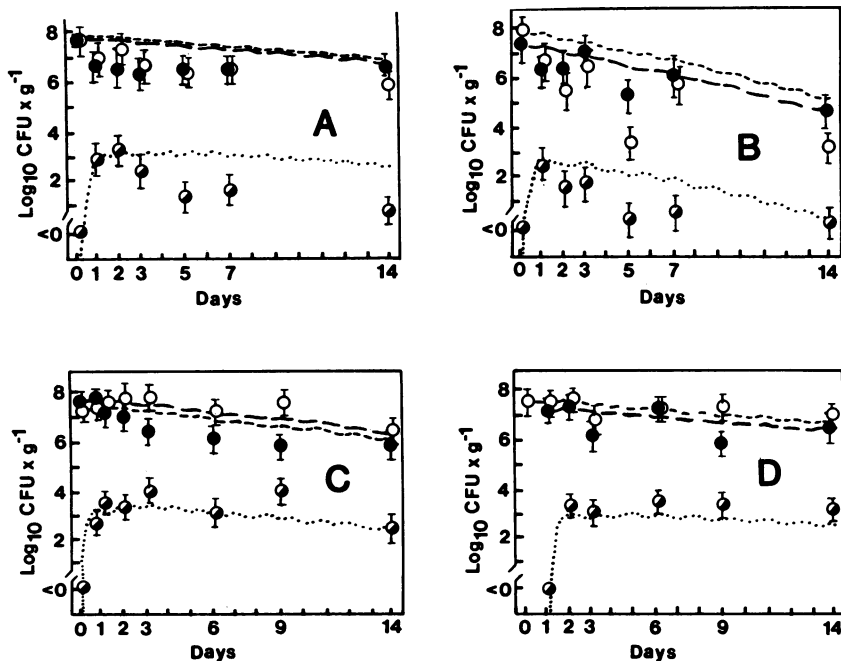


FIG. 2. Observed and predicted *P. cepacia* populations in phyllosphere microcosms in four experiments, on radish (A and B) or bean (C and D) plants. Predictions were made by calibrating the simulation model with observed populations at time zero, except in panel D, for which donor cells were added after 24 h. Symbols: ●, donor observed; —, donor predicted; ○, recipient observed; - - - - -, recipient predicted; ●, transconjugant observed;, transconjugant predicted. Confidence intervals (95%) around sample means are indicated by vertical bars.

Generally, after day 1 the total numbers of donors, recipients, and transconjugants were between 10 and 50% of total recoverable populations.

DISCUSSION

Natural systems are inherently complex, and it is difficult or impossible to design laboratory experiments that account for the multiple interactions in even the simplest of them. For example, it is obvious that test tubes and petri dishes differ from microcosms and that microcosms differ from fields of radish or bean plants in many physical, chemical, and biological parameters. A pragmatic approach is to use a model to identify and describe the main factors that determine system behavior at one level and then to make predictions about a higher-level system on the basis of these specific assumptions. In this study, we used this approach to predict dynamics of bacterial survival and plasmid transfer in soil-plant microcosms primarily on the basis of data obtained from laboratory experiments.

Quantitative prediction of growth and death of microorganisms in soil or on plant surfaces is a difficult problem. In our model, we chose to use simple exponential growth or decay functions to describe population dynamics of donor, recipient, and transconjugant strains in soil or on leaf surfaces. Such a model may be more appropriate for the relatively large initial population levels used in this study than for indigenous populations at low levels. For the rhizosphere microcosm, the exponential decay model was a good predictor of population dynamics over 7 to 12 days, although rates in the microcosm were somewhat lower than predicted. For longer periods, a more complex population model may be necessary, since there is evidence (8) that the rate of population decline changes over time, perhaps as a result of reduced competition for resources at lower population levels. Also, effects of starvation and stress on bacteria may become more pronounced over time. For bacteria on leaf surfaces, the model correctly predicted the declining population trends that were observed, but underestimated the large fluctuations on radish foliage. Bacterial populations on leaf surfaces fluctuate with small changes in relative humidity and leaf wetness within a canopy, as well as over the surface of an individual leaf (2, 7; Knudsen and Spurr, in press). However, simple exponential decay models similar to the one described here have been used to make reasonably accurate predictions of *P. fluorescens* and *P. cepacia* populations over 14-day periods under field conditions (2; Knudsen and Spurr, in press).

In most of the experiments, and especially in the phyllosphere microcosms, the model closely predicted the appearance of transconjugants within 1 to 2 days and their numbers at that time. Thus, our work would seem to support the general validity of the mass action plasmid transfer model described by Levin et al. (3) and to suggest that plasmid

transfer dynamics may have been similar under laboratory and microcosm conditions. However, results of our experiments with leaf disks did suggest that the plasmid transfer rate was not entirely independent of cell density. Linear regression of the log-transformed data indicated that the rate of plasmid transfer may have been higher at the lower cell densities tested. It is possible that some self-inhibitory effects were involved at the higher cell densities.

One area for further study might be the use of this methodology to evaluate plasmid transfer from introduced donor strains into resident strains of the same or different species. The model could be used to help design an appropriate sampling scheme and estimate necessary detection thresholds on the basis of predicted transconjugant appearance. Also, the model may be useful as a tool to further investigate effects of spatial distribution and adsorption onto surfaces on rates of plasmid transfer in nature and as a first step in predicting the frequency of more rare events, such as mobilization of nonconjugative plasmids in the rhizosphere or phyllosphere.

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