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A Continuous Culture Model to Examine Factors That Affect Transduction Among Pseudomonas Aeruginosa

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A CONTINUOUS CULTURE MODEL TO EXAMINE FACTORS THAT AFFECT TRANSDUCTION AMONG *PSEUDOMONAS AERUGINOSA*

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This dissertation examines factors that affect the accumulation of a novel bacterial phenotype in a population through the process of bacteriophage-mediated transduction. A mathematical model was created to predict changes in the relative number of transductants due to the formation of new transductants and competition with the existing population. Transduction was examined among *Pseudomonas aeruginosa* by quantifying the transfer of plasmid Rms149 from a plasmid-bearing donor to an F116-lysogenic recipient. Transduction was observed in continuous culture over a range of environmentally relevant generation times, concentrations, and donor-to-recipient ratios. Changes observed in the relative number of transductants in continuous culture were compared with the mathematical model. The rate at which transduction occurs was found to increase with the concentration of recipient bacteria and the phage-to-bacteria ratio.

To Paul

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CHAPTER I

INTRODUCTION

Environmental release of genetically engineered organisms has promising potential for applications in agriculture and biological remediation. The release of genetically engineered bacteria has led to questions regarding the persistence of these organisms and their genes in the environment. Ideally, the released organism should carry out its intended function without disrupting the balance of the natural ecosystem.

Bacteria vary their genetic makeup by undergoing processes of conjugation, transformation and transduction. Engineered genes of a recombinant organism may be transferred to members of the indigenous population. Organisms indigenous to a particular environment are likely to be better adapted for survival than introduced engineered bacteria. Therefore, if an engineered gene is transferred to an indigenous organism, its chances for stabilization in a bacterial population may increase.

This study examines factors that affect transduction, a gene transfer process mediated by viruses. There is evidence to indicate that aquatic bacteria are capable of undergoing

transduction (1, 42, 51, 52), but little is known about factors that influence transduction frequency. The objective of this dissertation research was to (i) create a mathematical model to describe how the processes of selection and transduction affect the proportion of transductants in a population and (ii) apply this model to continuous culture experiments in order to identify factors that influence transduction frequency. Experiments were performed in continuous culture so that the effects of concentration, donor-to-recipient ratio and generation time could be individually identified. This information could be used to predict how the rate of transduction of introduced genetic information may be influenced by the conditions of an environmental release.

The organism *Pseudomonas aeruginosa* was chosen for this study because of its wide distribution and well-characterized genome. Experiments were conducted in a chemostat under conditions that could occur in a freshwater environment. Transduction was ascertained by the transfer of plasmid markers from a plasmid-bearing donor to the plasmid-free, lysogenic recipient. The reasons for using a lysogen in this model are two-fold: (1) lysogeny imparts immunity from further phage infection, thus allowing for increased survival, and (2) spontaneous induction of lysogens into the lytic cycle provides a continual source of phage particles to the system (2, 56).

When the donor and recipient strains are co-incubated, plasmid transduction may occur by the following series of events (Figure 1). Free F116 phage particles are introduced into the system through the spontaneous induction of the recipient strain. A F116 phage particle adsorbs to and lyses a donor cell. Phage particles and plasmid containing transducing particles are produced. A transducing particle adsorbs to a recipient cell. The plasmid DNA is inserted, circularized and expressed, resulting in a transductant phenotype (56).

Changes in the proportion of transductants in the population were determined in experiments conducted over a range of cell concentrations, donor-to-recipient ratios and dilution rates. Apparent transduction rates were found to be dependent upon the concentrations of recipients and the phage-to-bacteria ratio.

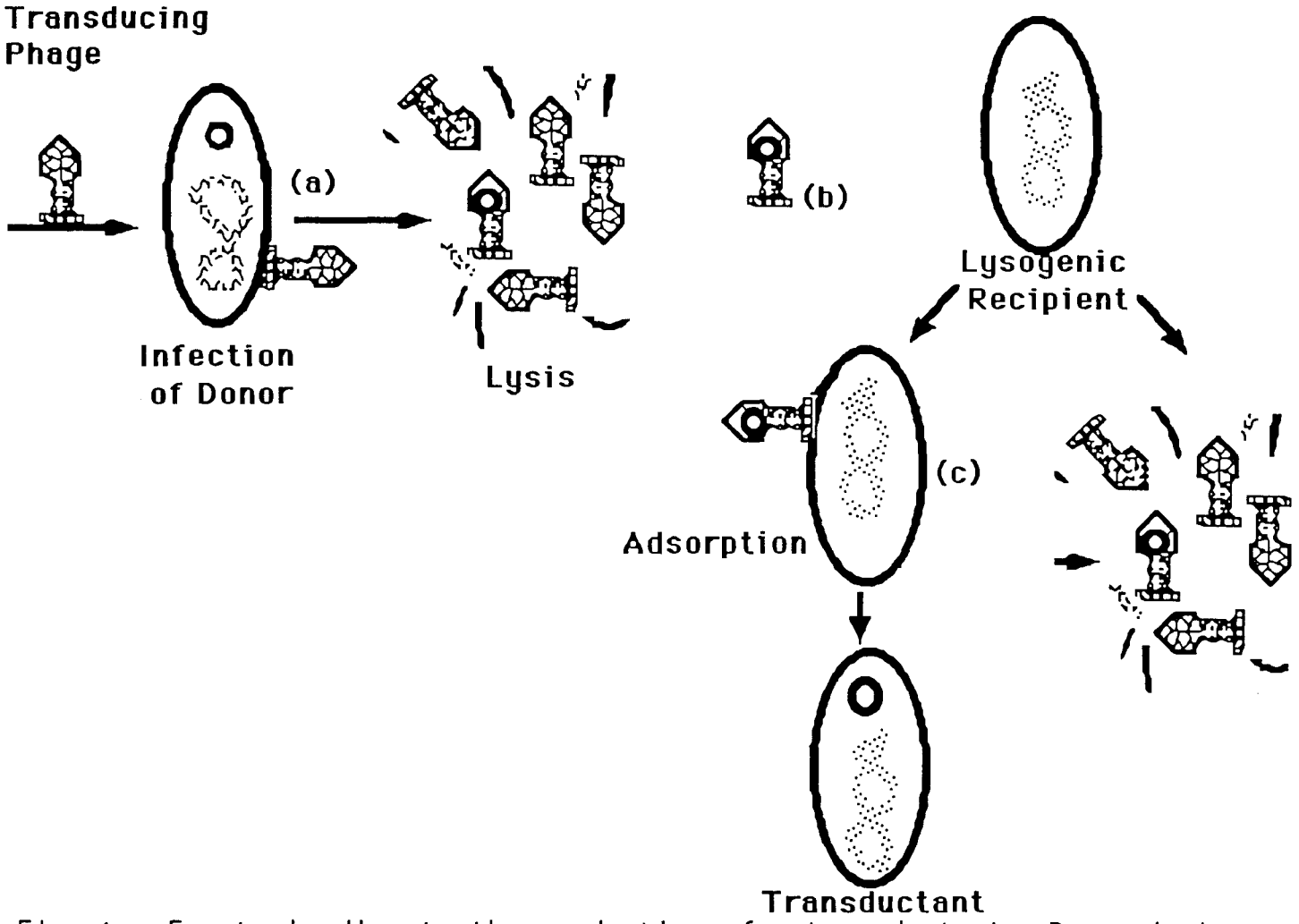


Fig. 1. Events leading to the production of a transductant. Bacteriophage adsorb to and lyses a donor cell (a), producing the occasional plasmid-bearing transducing particle (b). This particle adsorbs to and transduces the recipient (c). Spontaneous induction of the recipient supplies phage to the system (56).

CHAPTER II

REVIEW OF THE RELATED LITERATURE

Introduction

Pseudomonas aeruginosa, the organism chosen for this study, is a well characterized Gram-negative soil and aquatic bacterium (20). Because of its ability to metabolize a variety of organic compounds, it is widely distributed in natural environments (15).

P. aeruginosa strain PAO can be transduced by the temperate generalized transducing phage F116. Bacteriophage F116 adsorbs to the pili of strain PAO (53). The prophage DNA is maintained extrachromosomally as a plasmid (45).

Transduction

Transduction is a method of gene transfer in bacteria that is mediated by bacteriophages (27). It is contingent upon errors made during packaging of DNA into bacteriophage heads. Occasionally, bacterial DNA from the donor cell is mistakenly packaged into a phage capsid. The resulting transducing particle may then adsorb to a recipient bacterium and inject this bacterial DNA. If the DNA is incorporated into the genome of the recipient cell, the resulting

transductant may have an altered genotype. There are two types of transducing phages, generalized and specialized. With a generalized transducing phage, like F116 of *P. aeruginosa* (46), all areas of the host genome are incorporated equally. With a specialized transducing phage, such as the *P. aeruginosa* phage D3 (6), only segments of the host genome that are near the phage's chromosomal site of integration may be packaged.

Bacteria in the environment

Conditions for bacterial growth in the laboratory are usually optimized for enteric bacteria, such as *E. coli*. However, these growth conditions are not typical of the natural environment. Natural waters contain carbon concentrations of 1 to 15 mg/L, much lower than the 2 to 5 g/L found in many nutrient media (54). Roszak and Colwell (54) assert that slow growth at low carbon concentrations are normal conditions for bacterial growth in the environment. By culturing bacteria on rich media, one may actually prevent the growth of many environmental isolates (54). Also, Roszak and Colwell (54) propose that some bacteria exist as somnicells, cells that are viable but not culturable by traditional laboratory methods. Indeed, Jannasch (25) observed generation times of natural marine isolates ranging from 20-200 h long, with an average generation time of 53 h.

Estimates for environmental concentrations of bacteria depend upon the method of enumeration. Bacteria samples from unpolluted freshwater lakes and streams produce 10^3 to 10^4 colony forming units (CFU)/ml on nutrient agar plates (50, 56). *Pseudomonas* species from a Tennessee freshwater lake produce 7.0×10^1 to 1.0×10^3 CFU/ml on *Pseudomonas* Isolation agar (49).

However, plate count concentrations can be misleading because not all viable bacteria sampled may be able to grow on the chosen medium. Using the acridine orange direct count method, Lovell and Konopka measured cell concentrations of $2\text{-}6 \times 10^6$ cells/ml in an Indiana lake (40) and Brayton *et al.* found concentrations of 10^6 to 10^7 cells/ml in ponds and rivers of Bangladesh (4).

The aquatic environment is heterogeneous, varying in nutrient availability, pH and temperature with different locations and seasons (54). Bacterial concentrations are likely to be variable too, with higher concentrations congregating in microhabitats that are most favorable for growth (27).

Environmental concentrations of bacteriophage

Bacteriophages have been identified for almost all major groups of bacteria and can be found wherever bacteria grow (5). Apparent environmental concentrations of bacteriophages also depend upon the method of enumeration. Assaying for

bacteriophage plaques is the most common method used, but it relies upon the virus's ability to form plaques on the bacterial indicator strain. Bacteriophage able to attack enteric bacteria were found at concentrations of 5×10^{-2} to 1×10^{3} plaque forming units (PFU)/ml in a variety of freshwater environments (15). When indigenous freshwater species were used as bacterial hosts, 4×10^{-1} to 6 PFU/ml were observed in river water (15). The plaque assay method underestimates total bacteriophage concentrations because it overlooks phage that do not form plaques on the indicator strain(s) chosen.

Direct counts of total virus concentrations have been made using electron microscopy. Sewage samples observed by Ewert and Paynter (14) had concentrations of 2×10^{7} to 1×10^{8} virus particles/ml. These investigators found that only 6% of bacteria sampled from the same source could serve as hosts for the virus isolates (14). Bergh *et al.* (3) enumerated phage by centrifuging water samples directly onto electron microscope grids. Using this novel method, they observed concentrations of up to 2.5×10^{8} virus particles/ml in unpolluted marine waters. Water samples taken in winter were found to have a much lower concentrations of viruses ($<10^{4}$/ml), indicating a seasonal variation in virus concentration.

Although direct counts of bacteriophage can be made, this does not directly reveal what portion are capable of

transduction. Transducing phages have been found for numerous species of bacteria and are probably common in the bacterial world (27). In the environment or laboratory, the number of transducing particles that are successful in transducing and incorporating genetic sequences into new hosts are subject to several limitations. These include bacteriophage host range specificity, bacterial degradation of introduced DNA, and the breakdown of bacteriophage particles (27).

Transduction in aquatic microcosms

Transduction is used routinely as a means of mapping genes and constructing strains (6, 44). However, the importance of transduction as a mechanism of gene dispersal in nature is not well understood. Current information about the prevalence of phage-bacteria associations and the results of numerous microcosm experiments clearly show the potential for transductional gene transfer to occur in the environment (1, 47, 49, 51, 56, 57, 66).

Morrison *et al.* (47) studied transduction in *P. aeruginosa* using flow-through environmental test chambers suspended in a lake. They used a streptomycin-resistant F116 lysogen as the donor and source of phage. Transduction was observed to occur in these chambers at transfer frequencies of 1.4×10^{-5}, 5.9×10^{-5}, and 8.3×10^{-2} transductants per recipient after 1 hour, 4 days, and 10 days, respectively.

These rates included both primary transductants and daughter cells that arose from cell division of primary transductants. To estimate the frequency of actual transduction events, they examined changes in concentration of the total populations to approximate number of doublings. Transductants presumably underwent a similar number of doublings, provided that the they did not have a competitive advantage or disadvantage. A conservative transfer rate of 8.8 $x10^{-5}$ transductants per recipient was estimated (47).

Amin and Day (1) investigated F116-mediated transfer between two strains of *P. aeruginosa* that resided on membrane filters which were facing each other, and bound to river stones. The donor and recipient each carried two distinct antibiotic resistance markers. Transduction of the donor's markers to the recipient strain was observed. The frequency of transfer was dependent on cell density in laboratory trials but remained relatively constant *in situ* at 1.7 to 7.8 x 10^{-2} transductants per recipient.

Saye *et al.* (56) showed transductional transfer of plasmid Rms149 mediated by the *P. aeruginosa* phage F116L. Plasmid Rms149 is not capable of self-transmission. Highest transduction frequencies were found when they used a plasmid-bearing strain as the donor and a lysogen as the recipient and source of phage. In this paradigm, transduction occurred when the bacteriophage-lysed donor strain produced a transducing particle with plasmid DNA, which then adsorbed to

and transduced the recipient. *In situ* studies were performed in membrane diffusion chambers submerged in a lake. Plasmid transduction was shown both in the absence and presence of the indigenous community. The presence of the natural community caused a decrease in the concentration of introduced organisms and in the number of recoverable transductants. The donor-to-recipient ratio was found to influence the transduction frequency, with highest transductant recovery occurring at an intermediate donor-to-recipient ratio (20:1).

Saye *et al.* (57) were able to show *in situ* transduction of chromosomal markers as well. To be certain that the cells recovered were not merely revertants, they constructed strains of *P. aeruginosa* that allowed for the scoring of cotransduction of adjacent genetic markers. They demonstrated the ability of lysogens to serve both as the source of transducing phages and as the recipient of transduced information. Transductants of individual chromosomal alleles were retrieved at frequencies of 10^{-6} to 10^{-8} transductants per colony forming unit.

Osman and Gealt (51) demonstrated transduction in *E. coli* by indigenous transducing phages isolated from wastewater. They showed transfer of both plasmid and chromosomal DNA to *E. coli* $_{\chi}$1997. The recombinant plasmid pHSV106 could also be transferred to an indigenous isolate of

E. coli, which proved to be a better recipient than *E. coli* χ1997, yielding 10^{-4} versus 10^{-5} transductants per recipient.

Most of the previous studies on transduction were performed in closed microcosm systems, therefore, observed transduction rates were artificially high due to cell division of primary transductants. In order to determine the actual rate of transduction events, the number of cells resulting from cell division must be accounted for.

Lysogeny

Lysogeny is a semi-stable state for temperate bacteriophage, where prophage DNA is maintained and perpetuated along with the bacterial genome (33, 37). The prophage DNA may integrate into the host's genome, or exist as an extrachromosomal plasmid (37). In lysogeny, phage encoded repressor protein is produced to inhibit entrance of the prophage into the lytic cycle. Presence of repressor protein is advantageous to bacteria because it imparts immunity to reinfection by the same type of phage (61). However, temperate prophages are occasionally induced to enter the lytic cycle, lysing the bacterium and releasing many temperate progeny phages (37).

The lysogenic association of temperate phage with bacteria is also advantageous to the phage. Free phage particles are unstable in the environment and their concentrations drop rapidly over time (49, 56). Ogunseitan

et al. found that the half-life of *Pseudomonas* phage UT1 in natural lake water was 18 hours (49). Temperate phage, perpetuated by the occasional induction of a lysogen, provide a steady release of phage into a bacterial community (61). It provides the means for a more permanent establishment of the phage in a particular population by providing protection for the phage DNA and by preventing the extinction of the host (61). Levin and Lenski (37) propose that temperance may be an adaptation to low host-cell concentrations.

Paterson (52) found lysogeny to be very widespread in the genus *Pseudomonas*. Of the 31 strains of *P. aeruginosa* examined, 26 were found to be lysogenic. Holloway (20) estimates that most *P. aeruginosa* are lysogenic for at least one bacteriophage.

Ogunseitan *et al.* (49) conducted microcosm studies with natural isolates of *P. aeruginosa* in lake water using single or mixed populations of bacteriophages. Stable phage-bacteria relationships were maintained by the establishment of lysogenic or pseudo-lysogenic states in the host bacteria. In the lakewater microcosm, 45% of the cells became carriers of DNA from the introduced phage UT1 within 12 hours. After 45 days of incubation, 90% of the cells were carriers. They also examined environmental isolates of *P. aeruginosa* from their sampling site to look for possible natural associations of UT1. Forty-five percent of the isolates examined tested positive in colony hybridization with phage UT1 DNA.

Several factors may influence the establishment of lysogeny in a host. Starvation of *E. coli* before infection has been shown to increase the efficiency of lysogenization 50 to 100-fold (29). The establishment of lysogeny is also influenced by the ratio of phage to bacteria present. The frequency of lysogenization in lambda phage increases at higher multiplicities of infection (MOIs) and at least two infecting phages are required for the establishment of lysogeny (29). Several recombination-deficient(*recA*) mutants of *P. aeruginosa* have reduced efficiencies of lysogeny establishment by temperate phages D3, F116, and G101 (19, 43, 44). In these mutants, lysogeny may only be established at high MOIs (43).

Continuous cultivation in a chemostat

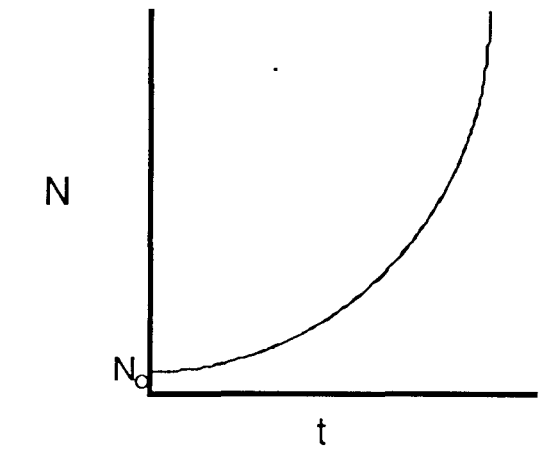
The chemostat is an ideal system for creating a microcosm model because it permits the continuous cultivation of organisms indefinitely in an unchanging environment (60). In batch culture, cells grow exponentially until nutrients are depleted and then enter a starvation period. By this time, metabolic by-products and dead cells have accumulated. In continuous culture, one is continually adding sterile nutrient medium and continuously removing exhausted medium, living cells, and cellular debris, thereby maintaining a constant volume. This allows for the maintenance of a steady state concentration of actively dividing cells (60).

In a steady state culture, one is able to separate and define parameters that are normally interdependent in batch fermentation, like cell density and growth rate (12). Cell density may be regulated by the concentration of the limiting nutrient provided in the medium. Cells equilibrate at the maximal cell density that the chosen medium can support. Specific cell growth rate is controlled by adjusting the rate of medium inflow. At equilibrium, the number of new cells from cell division will be balanced by the number of live cells that leave in the outflow. Therefore, with the chemostat, one can study population dynamics over a wide range of cellular densities and growth rates (60).

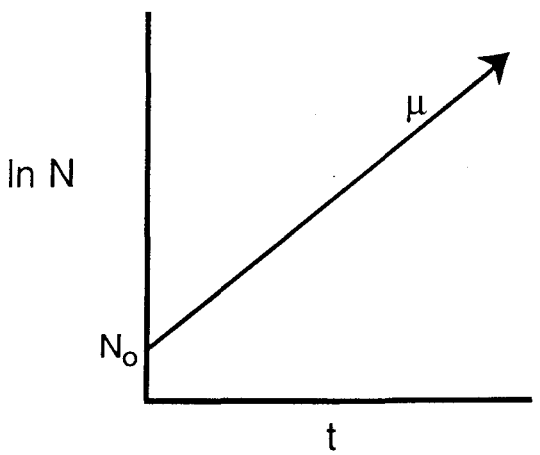
Cell growth rate

For a better understanding of a steady state chemostat, let us first consider batch culture growth in the presence of excess nutrients. Cell number increases in an exponential fashion as shown in Figure 2*a*. When this increase is plotted on a semi-log scale, as in Figure 2*b*, one may obtain a linear slope that describes the specific growth rate, μ, of organism (10). The increase in cell number N, with respect to time t is expressed as (30):

$$dN/dt = \mu N \qquad [1]$$



(*a*)



(*b*)

Fig. 2. Hypothetical illustration of non-limiting growth over time: (*a*) linear graph and (*b*) semi-log graph. *N*, cell number; N_O, initial cell number; *t*, time; μ, specific population growth rate (10).

The number of cells increases as:

$$N = N_o e^{\mu t} \quad [2a]$$

or $$N/N_o = e^{\mu t} \quad [2b]$$

The time it takes for cell number to double is termed the doubling time (T). It is given by (30):

$$N/N_o = 2 = e^{\mu T} \quad [3a]$$

or $$T = (\ln 2)/\mu \quad [3b]$$

where N is the cell number, μ is the specific growth rate and t is time.

Monod model of growth

As the substrate concentration of medium increases, so does the rate at which cells can grow, until this growth rate reaches a physiological maximum (Figure 3). The Monod model of growth describes the relation between cell growth rate and limiting substrate concentration in continuous culture. Based upon the Michaelis-Menten model for enzyme kinetics, the Monod model states (60):

$$\frac{\mu}{\mu_{max}} = \frac{S}{K_S + S} \quad [4]$$

where S is the substrate concentration in grams per liter, μ is the specific growth rate per hour, μ_{max} is the maximum

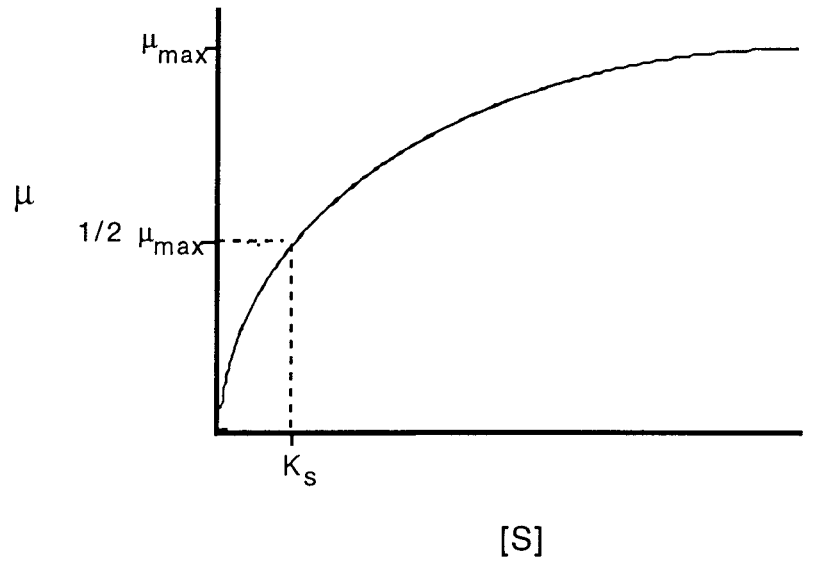


Fig. 3. The Monod relationship between substrate concentration and cell growth rate: [S], substrate concentration; μ, specific growth rate; μ_{max}, maximum growth rate; K_s, substrate concentration at 1/2 μ_{max} (30).

growth rate achievable on the substrate per hour, and K_S is the substrate concentration at which the specific growth rate is one-half of the maximum growth rate. The value for K_S is a measure of the efficiency with which a cell can procure substrate from its environment (30, 60).

Material balance on total cell biomass

In batch fermentation, there are events which alter the total cell biomass over time. Total cell mass in the vessel increases with cell division and with the entrance of any new cells in the incoming medium. Total viable biomass decreases with cells that washout or die. Therefore, from Stafford (60):

Accumulation = Incoming medium + Cell growth - Washout -Death

$$\frac{dX}{dt} = \frac{F}{V} X_O + \mu X - \frac{F}{V} X - aX \quad [5]$$

where dX/dt is the rate that total cell mass accumulates over time in grams of dry cell weight (DCW) per hour; X_o is the concentration of cells in the incoming medium in grams per liter; F is the flow rate of the chemostat in liters per hour; V is the volume of the reaction vessel in liters; μ is the specific growth rate for the culture per hour; X is the concentration of cells growing in the culture vessel at time t in grams of DCW per liter; and a is the specific rate of death of the culture per hour (60).

This equation may be simplified to describe cells in steady state equilibrium. At steady state, there is no net increase in cell mass, so $dX/dt = 0$. Unless there is contamination, there are no cells introduced in the incoming medium, so the term FX_o/V may be omitted. At steady state, cell death is considered negligible (60), allowing us to exclude the death term, aX. Therefore, the equation for a steady state culture is (60):

$$\frac{F}{V} X = \mu X \qquad [6a]$$

and

$$F/V = \mu \qquad [6b]$$

Because the chemostat volume is constant, the specific growth rate μ, can be regulated by altering the rate of flow of medium into the chemostat vessel. The specific growth rate may be varied up to the maximal growth rate that the organism can achieve.

The rate that the culture vessel is replenished with new medium is termed the dilution rate, D and is defined by,

$$\frac{F}{V} = D \qquad [7]$$

with the rate of dilution being expressed per hour (60). The time it takes for the vessel to be replenished is the culture generation time, τ , or,

$$\tau = \frac{V}{F} = \frac{1}{D} \qquad [8]$$

In one culture generation time, τ, one chemostat volume of media is added to the vessel and likewise, one chemostat volume leaves the vessel (30). However, this measurement does not represent the actual rate of cell division, since some cells are washed out of the chemostat vessel before they can divide and, if permitted, would divide after leaving the chemostat.

Cell generation time, T, refers to the rate of cell division (30). As described earlier, it is equivalent to rate at which total cell number increases by two-fold, or

$$N/N_o = 2 = e^{\mu T} \qquad [3a]$$

or

$$T = (\ln 2) \; / \; \mu \qquad [3b]$$

In continuous culture, cell growth is limited by the rate at which new medium is added to the vessel (30, 60). Therefore, the growth rate, μ, may be replaced by dilution rate, D, in Equation [3b]. Therefore, in continuous culture, cell generation time is given by (30):

$$T = (\ln 2) \; / \; D \qquad [9]$$

Selection in the chemostat

When culturing two or more bacterial strains in the chemostat, the less competitive strain(s) will be selected against and decrease in relative concentration over time (17). For example, a strain could be selected against due to a mutation which reduces the functional efficiency, or Km, of an enzyme required for cell metabolism. Selection results in a change in genotypic frequencies over time.

Hartl and Clark (17) provide a detailed explanation of selection. Consider two competing bacterial strains, *a* and *A*, which are grown together in a chemostat. If strain *a* has a faster growth rate, it will have greater relative fitness. Let w_1 and w_2 represent strain fitness, or the proportion of cells that survive after one generation. At generation t, strains *a* and *A* have frequencies of p_t and q_t, where $p_t + q_t = 1$. After one generation of selection has passed, a proportion w_1 of strain *a* and a proportion w_2 of strain *A* remain, so that

$$p_1/q_1 = p_o w_1/q_o w_2 = (p_o/q_o)(w_1/w_2) \qquad [10]$$

The relation between the ratios p_2/q_2 and p_1/q_1 would be the same as that between p_1/q_1 and p_o/q_o, so that

$$p_2/q_2 = (p_1/q_1)(w_1/w_2) = (p_o/q_o)(w_1/w_2)^2 \qquad [11]$$

This may be taken one step further to estimate the genotypic ratios for any generation t, that is:

$$p_t/q_t = (p_o/q_o)(w_1/w_2)^t \quad [12a]$$

or

$$\ln(p_t/q_t) = \ln(p_o/q_o) + t\ln(w_1/w_2) \quad [12b]$$

The term $\ln(w_1/w_2)$ is often expressed as the selection coefficient, s, so that (12, 60):

$$\ln(p_t/q_t) = \ln(p_o/q_o) + st \quad [13a]$$

or

$$(p_t/q_t) = (p_o/q_o)\, e^{st} \quad [13b]$$

A plot of $\ln(p_t/q_t)$ against t exhibits a linear relationship, with a slope of s. The value of s indicates how strain A, with genotypic frequency p_t, changes relative to strain a, with genotypic frequency q_t. Strain A has a selective advantage over strain a when $s > 0$, the relationship between strain a and A is neutral when $s = 0$ and strain a is has a selective advantage over strain A when $s < 0$ (12, 34).

The selection coefficient, s, is a measure of differential growth rate per unit time (12). Suppose that strain a had a growth rate of α, and strain A had a growth rate of β. The selection of strain a with respect to strain A would occur at a rate equivalent to the relative difference in growth rates between the two strains, or $(\alpha-\beta)/\beta$ (24).

Values for the selection coefficient, s, are expressed as selection per hour or selection per generation. Contrary to the discrete-generation models used in population genetics, Dykhuizen found that selection per generation increased with chemostat generation time (11). In his study, tryptophan auxotrophs were at a selective disadvantage with respect to wild-type *E. coli*. Selection against the auxotroph became greater as generation time increased, for generation times of up to 3 h. At generation times of greater than 3 h, selection against the auxotroph dropped to zero, indicating the absence of selection. Dykhuizen also observed that selection was independent of cell density (11).

Mathematical models of bacteriophage interactions

The kinetics of bacteriophage adsorption can be studied directly by combining known quantities of bacteria and bacteriophage, and measuring the decline in free phage over time (38, 59). From these data, one can obtain the adsorption rate constant, γ, using the formula,

$$\ln (\phi_o / \phi_t) = \gamma N t \qquad [14]$$

where ϕ refers to phage density, N is the number of bacteria, and t is time (59). Adsorption rates for *E. coli* T phages

are typically 10^{-7} to 10^{-8} contacts per milliliter per phage per cell per hour (33, 36, 38).

Population dynamics of bacteria and bacteriophage have been modeled after the law of mass action by studying the coexistence of *E. coli* and its virulent T phages (35, 38). Specifically, the mass action model predicts that a bacteriophage and host encounter each other randomly at a frequency that is jointly proportional to their concentrations. The number of encounters that result in successful phage attacks was described as $\delta N\phi$, where δ is the attack rate constant, N is the number of sensitive bacteria available and ϕ is the number of phage (35, 38). The attack rate constant, δ, can be estimated from the adsorption rate of virulent phage to host bacteria.

Levin (36) made a prediction for the frequency of generalized transduction in a population of *E. coli*. For his estimate, he used an average value of 5×10^{-8} for the adsorption rate constant (38, 59) and a value of 10^{-5} for the probability that a phage would be transmitting a particular gene (18). Multiplying these figures, he obtains a probability of 5×10^{-13} that a single phage particle will transfer a particular gene to a single bacterium in 1 ml in 1 hr. For a population containing 10^7 cells per ml and an equivalent number of phages, he estimates that transduction would occur at a rate of 5×10^{-6} per cell per hour. The

frequency would be less if the bacteria were resistant to the phage or if the transducing phage DNA was degraded by the host's restriction system (36).

Mutant Accumulation

Mathematical models of spontaneous mutant accumulation may prove to be useful for describing emerging transductant populations. Both mutant and transductant populations begin with just one cell and increase in number with the occurrence of new mutation or transduction events, respectively.

The accumulation of *E. coli* mutants resistant to bacteriophage has been studied in continuous culture (30, 31, 48). Under non-selective conditions the accumulation of mutant, m, in a parent strain N was described by

$$\frac{dm}{dt} = \frac{\lambda N}{T} \qquad [15]$$

where λ is the number of mutants produced per generation per bacterium and T is the cell generation time (48). Therefore, λ/T is an expression of the number of mutants produced per unit of time per bacterium. The number of mutants that accumulate at any time t is:

$$m = m_0 + \frac{\lambda N t}{T} \qquad [16]$$

From this equation, one can see that the rate of mutant accumulation is linear with time. To calculate the rate of mutant accumulation, one plots the concentration of mutants against time. This gives the slope, $(\lambda/T)N$, which may then be divided by the concentration of the parent strain, N, to obtain the rate of mutant accumulation per unit of time, λ/T.

Factors that may influence transduction

The rate of transduction may be influenced by the concentrations of the interacting components and the ratios between them. As described in a previous section, Levin (36) suggests that the rate of transduction will be dependent upon bacteria and bacteriophage densities. Zeph *et al.* (66) found that the frequency of transduction of *E. coli* by a cell-free lysate of bacteriophage P1 increases as the MOI increases from 0.3 to 9.5. Saye *et al.* (56) observed highest frequencies of transduction at an intermediate ratio of plasmid-bearing donors to lysogenic recipients. Because the ratio of nonlysogenic donors and lysogenic recipients would cause differences in the MOI, they suggest that an optimal MOI for transduction was achieved at this intermediate donor-to-recipient ratio.

Increased rates of transduction have also been found when the recipient bacteria are lysogenic for the transducing

bacteriophage (2, 56, 57). Lysogens may still be transduced but are immune to superinfection by that bacteriophage, thus enhancing their ability to survive. Benedik *et al.* (2) found 10- to 100-fold greater frequencies of F116 transduction in *P. aeruginosa* when using a F116 lysogen as the recipient. In their studies on F116-mediated transduction in *P. aeruginosa*, Saye *et al.* (56, 57) examined frequencies of gene transfer when (i) the donor is lysogenic and the recipient is nonlysogenic (ii) the donor is nonlysogenic and the recipient is lysogenic and (iii) the donor and recipient are both lysogenic. They observed highest transduction frequencies when a lysogen was used as the recipient and a non-lysogen was used as the donor. In these closed chambers, the spontaneous induction of lysogens provided a continual source of transducing particles to the system.

When considering the possibility of a gene transfer event occurring between genetically engineered bacteria and the indigenous population, Lenski (32) discusses the importance of considering the fitness of the recombinant organism and gene. The likelihood of a recombinant gene being transferred is dependent upon the persistence of the recombinant organism in the environment. The more fit a released organism is, the greater the chance of its persistence and the transfer of its engineered genetic material. However, it is necessary that the released

organism be fit enough to perform its intended biotechnological function. Also, the length of time that a recombinant organism can persist in the environment would depend upon the scale of its release (32). Lenski (32) states that an engineered gene transferred to an indigenous organism will be consequential only if it provides some selective advantage.

CHAPTER III

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages

All *P. aeruginosa* strains, their roles in this study and relevant genotypes are listed in Table 1. The *P. aeruginosa* plasmid Rms149 is non-mobile (Tra^- Mob^-) and carries genes for resistance to carbenicillin, gentamicin, streptomycin, and sulfonamide (56). The generalized transducing bacteriophage F116 (21) has been shown to efficiently transduce plasmid Rms149 DNA (56).

Media and cell growth

Unless otherwise indicated, bacteria used in all routine laboratory procedures were grown in Luria broth or on L-agar plates (44). When required for selection, antibiotics were used in the following concentrations: rifampicin, 75 µg/ml; carbenicillin, 500 µg/ml; nalidixic acid, 500 µg/ml; streptomycin, 1000 µg/ml; and chloramphenicol, 200 µg/ml. Transductants were selected on L-agar containing higher concentrations of rifampicin (600 µg/ml) and carbenicillin (1000 µg/ml) to discourage growth of revertants. Stock solutions of nalidixic acid (Sigma, St. Louis, MO) were

TABLE 1

Characteristics and roles of *Pseudomonas aeruginosa* strains used in the model

Strain	Role in model	Relevant characteristics[a]	Plasmid	Source or Reference
PAO1	INDICATOR	Prototroph	none	33
PAT1	INDICATOR	Prototroph	none	14
RM2141	PRECURSOR OF DONOR	*met-9011 nalA5 amiE200*	Rms149	46
RM2235	DONOR	*nalA5 amiE200*	Rms149	This study
RM300	RECIPIENT	*rif-901 chl-901* F116 lysogen	none	This study
RM4412	TRANSDUCTANT	*rif-901 chl-901* F116 lysogen	Rms149	This study
RM287	MOCK RECIPIENT	*rif-901 chl-901*	none	This study
RM289	MOCK TRANSDUCTANT	*rif-901 chl-901*	Rms149	This study

[a]Genotype symbols follow the conventions recommended by Demerec *et al.* (8).

prepared in 0.01 N NaOH at 50 mg/ml. Rifampicin (Sigma) was dissolved in methanol to 75 mg/ml before use.

Those experiments requiring selection on minimal medium agar were done using *Pseudomonas* Minimal Medium (PMM) (44) supplemented with 0.4% (w/v) glucose and 1.8% (w/v) Bacto-agar. Chemostat experiments and batch studies were performed using a variation of *Pseudomonas* Minimal Medium where the sodium citrate was omitted (PMM-c). Sodium citrate was excluded because it was found to serve as a source of carbon. The medium was supplemented with yeast extract as the sole nutrient source. Selection for the ability to use acetamide as a sole carbon and nitrogen source (Ami^+) was performed on PMM-c agar containing 0.01 M acetamide.

Cultures grown for routine laboratory procedures were incubated at 37°C. Chemostat and batch culture studies were carried out at room temperature to represent conditions that may occur in the environment. Liquid culture growth of *P. aeruginosa* was monitored in Klett-Summerson flasks which permitted the reading of $Klett_{660}$ values using a Klett-Summerson Photoelectric Colorimeter (Klett Mfg. Co., New York, NY).

Bacteriophage titration and lysate preparation

Bacteriophage titrations were prepared by combining a sample of the desired dilution of the phage preparation, 0.1-0.2 ml of indicator bacteria in mid-exponential growth phase,

and 2.5 ml of lambda top-agar (45). This mixture was poured over L-agar plates, incubated overnight at 37°C, and examined for the number of plaque-forming units (PFU).

Cell-free phage lysates were prepared in a similar manner, using 0.1-0.2 ml of an overnight culture of the host bacterium and approximately 10^7 PFUs per plate. After overnight incubation, the phage were harvested by removing the top-agar to a screw-cap tube. The L-agar was rinsed with 1.5 ml LB, which was added to the top-agar. One-tenth volume of chloroform was added to the tube to lyse the bacteria. The mixture was shaken vigorously and centrifuged for 20 min at 5,000 rpm in a Sorvall GLC-2B centrifuge. The supernatant fluid was filtered through a 0.45 µm filter, titered, and stored at 4°C.

Transductions

Transductions were performed as described by Miller and Ku (44) to construct strains and to evaluate media for optimal transductant recovery. For the transductions, cell-free F116 lysates were prepared on the desired strain as described in a previous section. The strain to be transduced was grown to late-exponential phase and infected with phage over a range of multiplicities of infection (MOI), distributed around an equivalent number of phage and bacteria. Cell-free lysates of F116 were added at concentrations to give MOIs of 100.0, 10.0, 1.0, 0.1, and

0.01. The transduction mixtures were spread onto selective media and incubated for 1-2 days at 37°C.

Preparation of plasmid DNA

Plasmid DNA minipreparations used for transformation and for verification of the transductant genotype were carried out using the alkaline-lysis procedure described by Maniatis (41). The protocol was followed as indicated with the exception that the phenol:chloroform extraction of DNA was performed twice.

Construction of strains

Several bacterial strains were constructed for use in the transduction system. The plasmid-donating strain, RM2235, was constructed from the strain RM2141 (57). Strain RM2141 carries plasmid Rms149 and has distinctive chromosomal markers including nalidixic acid resistance (*nalA5*), methionine auxotrophy (*met-9011*), and the inability to grow on acetamide as a sole source of carbon (*amiE200*). Strain RM2235 was constructed by transducing RM2141 to methionine prototrophy using a F116 lysate made on *P. aeruginosa* PAO1 and selecting for transductants on *Pseudomonas* Minimal Medium plates.

The plasmid-free nonlysogen, RM287, was obtained by isolating a spontaneous mutant of *P. aeruginosa* PAO1, resistant to the antibiotics rifampicin (*rif-901*) and

chloramphenicol (*chl-901*). A resistant mutant was first selected on rifampicin (75 µg/ml) and then used to select for a chloramphenicol resistant mutation (200 µg/ml). Strain RM300 is a F116 lysogen of RM287, and was obtained by spotting a bacteriophage F116 lysate onto a top-agar overlay of RM287. The lysogenic variant was selected for its ability to resist super-infection and thus grow in the area where the phage lysate had been spotted.

Strain RM289 (mock-transductant), was constructed by transforming RM287 with plasmid Rms149 using the $MgCl_2$ transformation procedure for *P. aeruginosa* (42). Transformants were selected for plasmid-encoded carbenicillin resistance.

Determination of cell densities in batch culture

Batch culture experiments were performed in PMM-c supplemented with a range of yeast extract concentrations to determine the resultant cell densities. Thirty-three tubes containing 1 ml of PMM-c were supplemented with yeast extract to give final concentrations ranging from to 1×10^{-9} % to 0.1% (w/v) yeast extract. The tubes were inoculated with 0.01 ml of an exponential-phase culture to concentrations of 10^2, 10^3, or 10^4 cells/ml, using less cells for lower dilutions of yeast extract. The tubes were incubated with at 200 rpm for 2 days at 37°C. The resulting cell densities were determined on L-agar plates.

Growth Curves

Maximal growth rates were determined by monitoring changes in cell density in growing cultures using the Klett-Summerson Photoelectric Colorimeter. Duplicate flasks containing 10 ml of PMM-c supplemented with 0.5% yeast extract were inoculated to approximately 5 $Klett_{660}$ units with the bacterial strain. Frequent readings of the $Klett_{660}$ values were made during growth, until the cultures reached approximately 100 $Klett_{660}$ units. The maximal specific growth rates were taken from the exponential period of cell growth.

Determination of transducing-particle-to-total-phage ratio

Batch experiments were performed at various donor-to-recipient ratios to determine the number of transducing particles per bacteriophage. Flasks containing 50 ml of PMM-c with 0.5% yeast extract were inoculated with exponential phase cultures at donor-to-recipient ratios from 42:1 to 1:244 to give a total concentration of 3×10^6 cells/ml. The flasks were incubated at room temperature on a rotating platform at 200 rpm. At 16 h and 25 h, the flasks were assayed for concentrations of recipient, donor, bacteriophage, and transducing particles.

The transducing particle concentrations were determined in the following manner. Ten milliliters of the culture sample was spun for 20 min at 5,000 rpm in a Sorvall GLC-2B centrifuge. The supernatant fluid was filtered through a

0.45 μm filter to exclude any remaining bacteria and used to transduce an exponential phase recipient strain at an MOI of 0.1. The transduction mixture was spread on selective plates and incubated two days at 37°C. The number of transductant colonies that appeared was used as an indication of the number of transducing particles present in the original sample.

Preparation and inoculation of chemostat

The Bioflo C32 fermenter (New Brunswick Scientific, Edison, NJ) was used for all chemostat experiments. In preparation for each run, 10 liters of PMM-c supplemented with the desired concentration of yeast extract was autoclaved with the chemostat reactor vessel and attachments. After sterilization, the chemostat reactor vessel was filled with medium.

The chemostat was inoculated in the following manner. Overnight cultures of bacteria were grown in LB under the appropriate selection. These cultures were diluted to 10 $Klett_{660}$ units in PMM-c supplemented with 0.5% yeast extract. When the cultures reached mid-exponential phase (55 $Klett_{660}$ units), cells were washed once with the chemostat medium and used to inoculate the chemostat. All trials were conducted aerobically at room temperature with an agitation rate of 200 rpm.

Procedure for transduction chemostat experiments

Transduction experiments were inoculated with the recipient strain, RM300, and the donor strain, RM2235 at concentrations indicated in the specific experiments. In initial transduction trials, the nutrient pump was turned on to the desired dilution rate at the time of inoculation. In later experiments, there was an initial 48 h batch period before the pump was activated. This batch period allowed transductants to accumulate to a detectable level so that changes in their densities could be monitored.

At least once a day, the chemostat was sampled and bacterial concentrations were determined on L-agar selection plates as follows: recipients on rifampicin, donors on nalidixic acid, Rms149 containing cells on carbenicillin, and transductants on rifampicin and carbenicillin. Also, a total cell count was performed on L-agar alone. To obtain significant numbers of transductants, it was necessary to pellet 15 to 200 ml in a Sorvall GLC-2B centrifuge for 10 min at 5,000 rpm before performing plate counts. The number of bacteriophage were determined by titering the supernatant fluid, using *P. aeruginosa* PAO1 as indicator strain or *P. aeruginosa* PAO1 and PAT1 in experiments where the cryptic phage was present.

Procedure for control chemostat experiments

Control experiments were peformed using PMM-c

supplemented with 4 x 10^{-5}% yeast extract, resulting in donor (RM2235) and mock-recipient (RM287) concentrations of 10^5-10^6 cells/ml. The mock-transductant strain (RM289) was inoculated at approximately 10^1 cells/ml. The nutrient pump was turned at the time of inoculation. The chemostat was sampled for concentrations of mock-recipient, donor, and mock-transductant in the same manner as described above. Although none were expected, the sample was also assayed for bacteriophage by titering the supernatant fluid, using *P. aeruginosa* PAO1 as the indicator strain.

Verification of transductants

The identity of transductants isolated in chemostat experiments was verified by replica plating transductants onto other selective media. Transductants were confirmed by their ability to grow in the presence of chloramphenicol and streptomycin and on PMM-c plates with acetamide as the sole carbon source. Transductants were additionally verified by their inability to grow on L-agar containing nalidixic acid. Selected transductants were examined further by performing plasmid minipreparations, which were restriction digested and analyzed by agarose gel electrophoresis.

Restriction digestions and gel electrophoresis

Minipreparations of plasmid DNA were digested with *Eco*R1 (Boehringer-Mannheim, Indianapolis, IN) using the conditions

suggested by the manufacturer. The digestions were terminated by the addition of tracking dye consisting of 30% (w/v) glycerol, 7% (w/v) sodium lauryl sulfate, and 0.07% (w/v) bromophenol blue. The DNA fragments were separated by electrophoresis through an agarose gel containing 0.7% (w/v) agarose and 0.7 µg/ml ethidium bromide in TBE buffer. TBE is composed of 0.089 M Tris-base, 0.089 M boric acid, and 0.002 M EDTA (pH 8.3) (15). The DNA samples were loaded into wells, including one well of *Hin*dIII-digested lambda DNA as a molecular-weight marker. A constant voltage of 100 V was applied to the gel until the bromophenol blue dye front had traveled at least 100 mm. The gel was visualized on a UV transilluminator (Ultraviolet Products, Inc., San Gabriel, CA) and photographed.

Identification of lysogens

Donors obtained from the transduction experiments were examined to determine the percentage which had become lysogens, as evidenced by the spontaneous induction of infectious phage particles from F116 prophage. This was accomplished by transferring eight to twenty-four colonies into the wells of microtiter plates containing approximately 0.2 ml of LB in each well. These were incubated with shaking for at least 5 h at 37°C. The microtiter plates were then spun in a Sorvall GLC-2B centrifuge for 10 min at 4,000 rpm. Five microliters of supernatant fluid from each well were

spotted onto a top-agar overlay of *P. aeruginosa* PAO1. After overnight incubation at room temperature, the plates were examined for phage clearing of the indicator strain.

Statistical methods

Significance between experimental parameters was determined by linear regression analysis using the statistical program, SYSTAT (1989, Systat Inc., Evanston, IL). The null hypothesis was rejected, and the correlation considered significant, at p values (probability) of 0.05 or less. Bacterial growth rates, and their standard errors, were determined from the coefficients obtained in linear regression analysis.

Multiple linear regression analysis was performed to determine the significance between experimental parameters and the transduction rate. A multiple linear regression model tests the significance of more than one variable on a dependent variable. Squared multiple correlations (R^2) and p values were computed for each multiple linear regression model.

Values for the selection coefficient and transduction rate were determined by using experimental data for the variables in Equations [17] and [25] and solving for the best fit values of s and $\Delta(T/N)_{trans}$. These values, and their standard errors, were calculated with the nonlinear modeling function of SYSTAT. This function begins with an initial

estimate of the parameter value and modifies it in small steps until the fit of the curve to the data is as close as possible. The best fit is determined by nonlinear least squares, which minimizes the squared deviation of the dependent variable values from values estimated by the function at the same independent variable data points.

CHAPTER IV

CREATING A MATHEMATICAL MODEL

Introduction

In order to understand the dynamics of the transductant phenotype in the continuous culture experiments, a two-part model was created to separate and identify the effects of selection and transduction. The selection term describes the change in the relative concentration of transductants due to negative or positive selection. The transduction term describes the rate at which transduction events add new transductants to a population.

Selection

As described in the Literature Review, competition in continuous culture may be described by the change in the ratio of two competing genotypes, or

$$p_t/q_t = (p_o/q_o)(w_1/w_2)^t \qquad [12a]$$

or

$$\ln(p_t/q_t) = \ln(p_o/q_o) + t\ln(w_1/w_2) \qquad [12b]$$

where p and q are the frequencies of the genotypes, w_1 and w_2 are their respective fitnesses, and t is time in generations or hours (17). The term $\ln(w_1/w_2)$ is a measure of the

relative difference in fitness and is often expressed as the selection coefficient, s, or (7, 12, 34),

$$\ln(p_t/q_t) = \ln(p_o/q_o) + st \qquad [13a]$$

In the control experiments, the transductant phenotype is a minor component of the total population. Its concentration does not contribute significantly the total cell density. Therefore, the ratio of transductants to non-transductants can be approximated as the frequency of transductants per total. Selection of transductants may then be described as

$$\ln(T/N) = \ln(T_o/N_o) + st \qquad [17a]$$

or

$$(T_t/N_t) = (T_o/N_o)\, e^{st} \qquad [17b]$$

where T represents the concentration of transductants, N is the total concentration and s is the selection coefficient. Equation [17] states that for any time, t, the relative proportion of transductants to total cells changes from the initial ratio by a factor of e^{st}.

The value for the selection coefficient, s, will be positive or negative depending on whether the transductant phenotype, T, is more or less fit than the majority phenotype, represented by N. Values for the selection coefficient may be empirically determined from changes in the relative concentrations of these populations over time.

Transduction

To model population dynamics in transduction experiments, it was necessary to develop an expression to describe how the frequency of transductants would change when both selection and transduction were occurring. Equations were formulated to describe how the transductant frequency would be expected to change after each generation.

With selection alone, the relative frequency of two strains change each generation as described by (17),

$$p_2/q_2 = (p_1/q_1)(w_1/w_2) \qquad [10]$$

where p and q represent the concentrations of two competing populations, and w_1 and w_2, are their respective fitnesses (see Literature Review). Equation [10] was modified for the transduction experiments, where transductants, T, make up a minor component of the total population, N. Let f represent the selection factor, or the fitness of transductants relative to the rest of the population. Therefore, values of f greater than one would indicate positive selection, whereas values of f less than one would signify negative selection. With selection alone, the transductant-to-total ratio would change after each generation as

$$(T/N)_2 = (T/N)_1 f \qquad [18]$$

Therefore, the selection factor, f, relates to the selection coefficient s, in the following way:

$$s = \ln f \qquad [19]$$

A transduction term was added to Equation [18] to include the addition of newly formed transductants in the population. The relative increase in transductants from selection and transduction after each generation would be

$$(T/N)_2 = (T/N)_1 f + \Delta(T/N)_{trans} \qquad [20]$$

where $\Delta(T/N)_{trans}$ is the increase in the number of transductants per total per generation due to transduction. The term $\Delta(T/N)_{trans}$ is constant when the donor and recipient populations are stable and present at much greater concentrations than transductants. Under these conditions, the transducing particle density is constant and transduction does not cause significant changes in the recipient concentration.

Hypothetical values of $(T/N)_o$, f, and $\Delta(T/N)_{trans}$ were input into Equation [20]. Transductant-to-total ratios were calculated for 50 generations using the BASIC program given in the Appendix. Changes in the transductant-to-total ratio were computed for various values of initial ratio, $(T/N)_o$, selection factor, f, and transduction rate, $\Delta(T/N)_{trans}$. The individual effects of each variable were examined.

Conditions of positive selection (f >1) resulted in an continuously increasing proportion of transductants in the population. This increase was more rapid as the value for selection factor increased (Figure 4). Both positive selection and the formation of transductants acted to increase the proportion of transductants in the population. If all members of the population have identical nutritional requirements, positively selected transductants could eventually outcompete the other bacterial strains. Under neutral selection (f = 1), the transductant-to-total ratio increases linearly at a rate equivalent to the relative rate of transduction, $\Delta(T/N)_{trans}$ (Figure 4).

Under conditions of negative selection (f <1), the transductant-to-total ratio was found to approach a limit at which transductants stabilize relative to the rest of population. The limiting transductant-to-total ratio, $(T/N)_{\infty}$, was influenced by the selection factor (Figure 5) and transduction rate (Figure 6). Observations of limiting transductant-to-total ratios, $(T/N)_{\infty}$, revealed the following relationship with selection factor, f, and transduction rate, $\Delta(T/N)_{trans}$:

$$\lim_{i \to \infty} (T/N) = \frac{\Delta(T/N)_{trans}}{1-f} \qquad [21]$$

In addition, transductant-to-total ratio limit appeared to be approached in a first-order exponential manner.

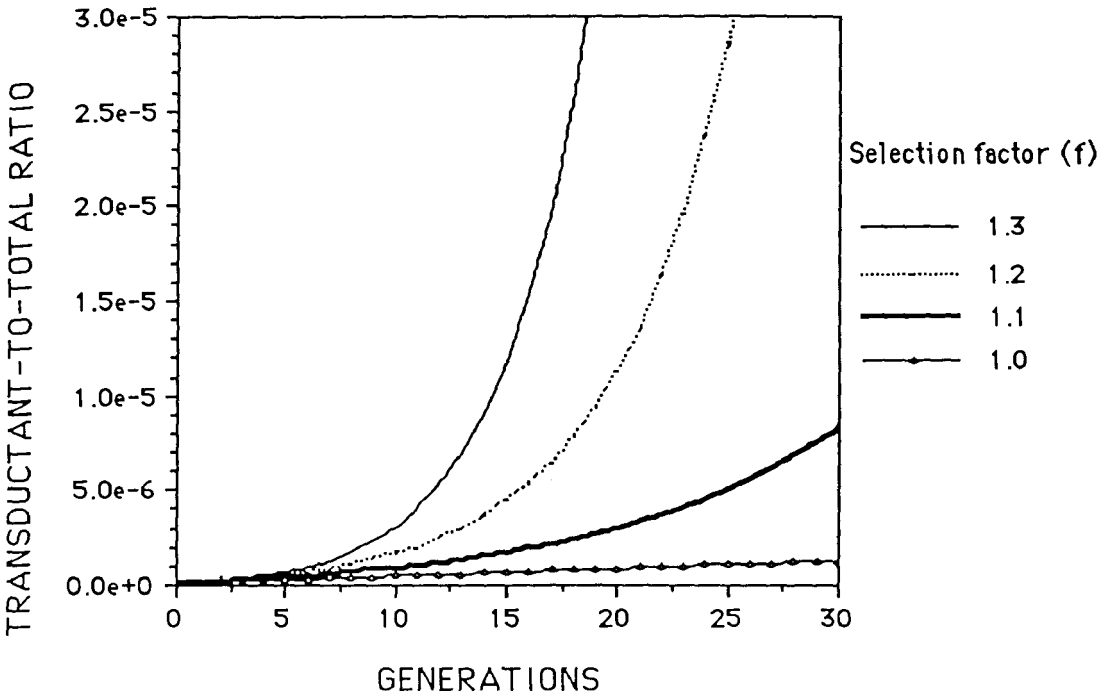


Fig. 4. Calculated changes in the transductant-to-total ratios for conditions of positive selection. Ratios were calculated from Equation [20] using an initial transductant-to-total ratio, $(T/N)_o$, of 1×10^{-7} and a transduction rate, $\Delta(T/N)_{trans}$, of 4×10^{-8} transductants formed per total per generation. Under positive selection, transductant-to-total ratios increased continuously and did not reach a limit.

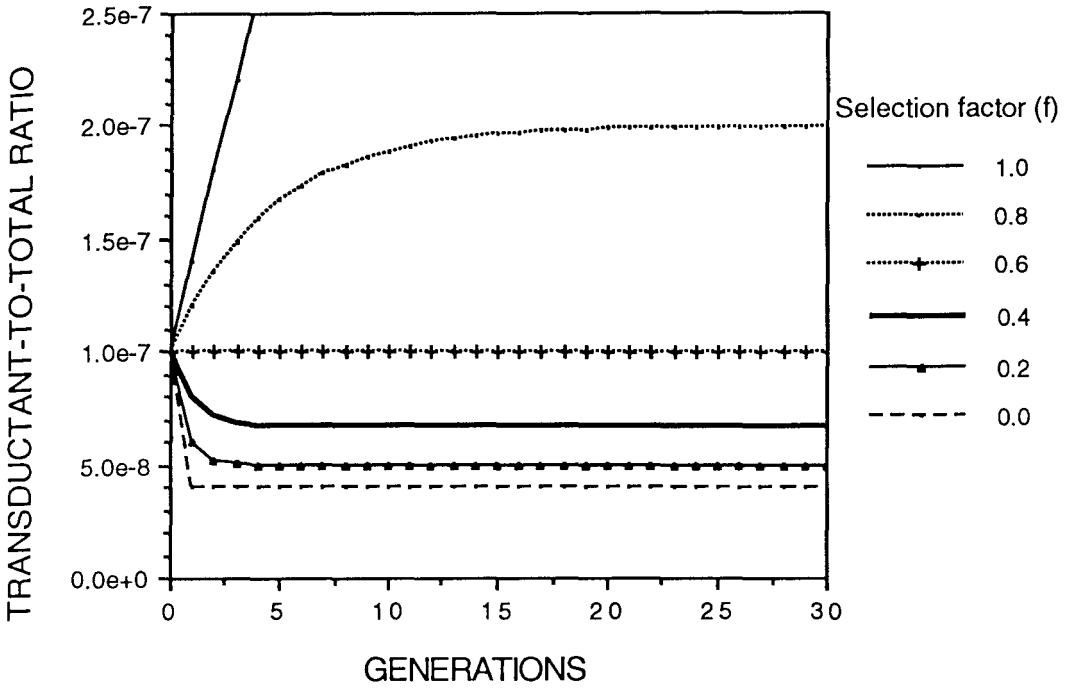


Fig. 5. Calculated changes in the transductant-to-total ratios for conditions of negative selection. Ratios were calculated from Equation [20] using an initial transductant-to-total ratio, $(T/N)_o$, of 1×10^{-7} and a transduction rate, $\Delta(T/N)_{trans}$, of 4×10^{-8} transductants formed per total per generation. Under negative selection, transductant-to-total ratios increase or decrease until reaching a limiting value, as described by Equation [21].

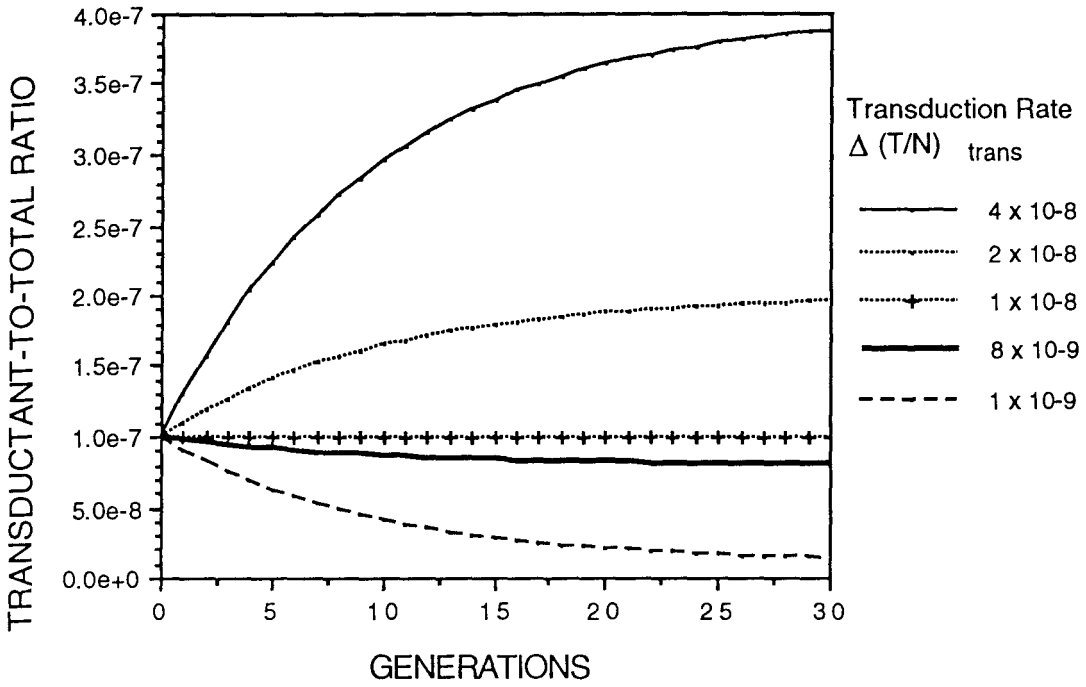


Fig. 6. Calculated changes in the transductant-to-total ratios over a range of transduction rates. Ratios were calculated from Equation [20] using an initial transductant-to-total ratio, $(T/N)_o$, of 1 x 10^{-7} and a selection factor, f, of 0.9. The transductant-to-total ratios increase or decrease until reaching a limiting value, as described by Equation [21].

The selection factor was then varied for conditions of negative selection. The calculated transductant-to-total ratios either increased or decreased from the initial ratio, until reaching the limiting ratio as given by Equation [21] (Figure 5). The value of the limiting ratio increased with the selection factor, f. Under complete negative selection (f =0), transductants are predicted to stabilize at a ratio equivalent to the relative number of transductants formed per generation, $\Delta(T/N)_{trans}$.

Increasing the transduction rate also resulted in higher limiting values of the transductant-to-total ratio (Figure 6). The ratio either increased or decreased from the its initial value to approach the limiting value as given by Equation [21].

The initial proportion of transductants in the population does not affect the transductant-to-total limiting ratio (Figure 7). Independent of the initial ratio, $(T/N)_o$, the transductant-to-total ratio increased or decreased to approach the limit as described by Equation [21].

Given a limiting value, as in cases of negative selection, one may describe the exponential change in the transductant-to-total ratio in terms of a first-order reaction (64), that is,

$$\frac{(T/N)_\infty - (T/N)_g}{(T/N)_\infty - (T/N)_o} = e^{\ln(f)g} \qquad [22]$$

When this equation is solved for $(T/N)_g$, one obtains:

$$(T/N)_g = (T/N)_\infty - [\ (T/N)_\infty - (T/N)_o\ ]\ e^{\ln(f)g} \qquad [23]$$

Taking the natural logarithm, and rearranging Equation [23], permits the equation to be expressed in a linear form, or

$$\ln[(T/N)_\infty - (T/N)_g\ ] = \ln(f)g + \ln[(T/N)_\infty - (T/N)_o] \qquad [24]$$

Equation [24] indicates that values of $\ln[(T/N)_\infty - (T/N)_g\ ]$ plotted against generations, g, gives a linear slope equivalent to $\ln f$ (Figure 8).

If the term $(T/N)_\infty$ in Equation [23], is substituted with the right side of Equation [21], we obtain,

$$(T/N)_g = \frac{\Delta(T/N)_{trans}}{1-f} - \left[\ \frac{\Delta(T/N)_{trans}}{1-f} - (T/N)_o\ \right] e^{\ln(f)g} \qquad [25]$$

Given experimental values for transductant concentration, total concentration, generations and selection factor, Equation [25] may be solved for $\Delta(T/N)_{trans}$. The selection factor may be determined from control experiments where transduction cannot occur.

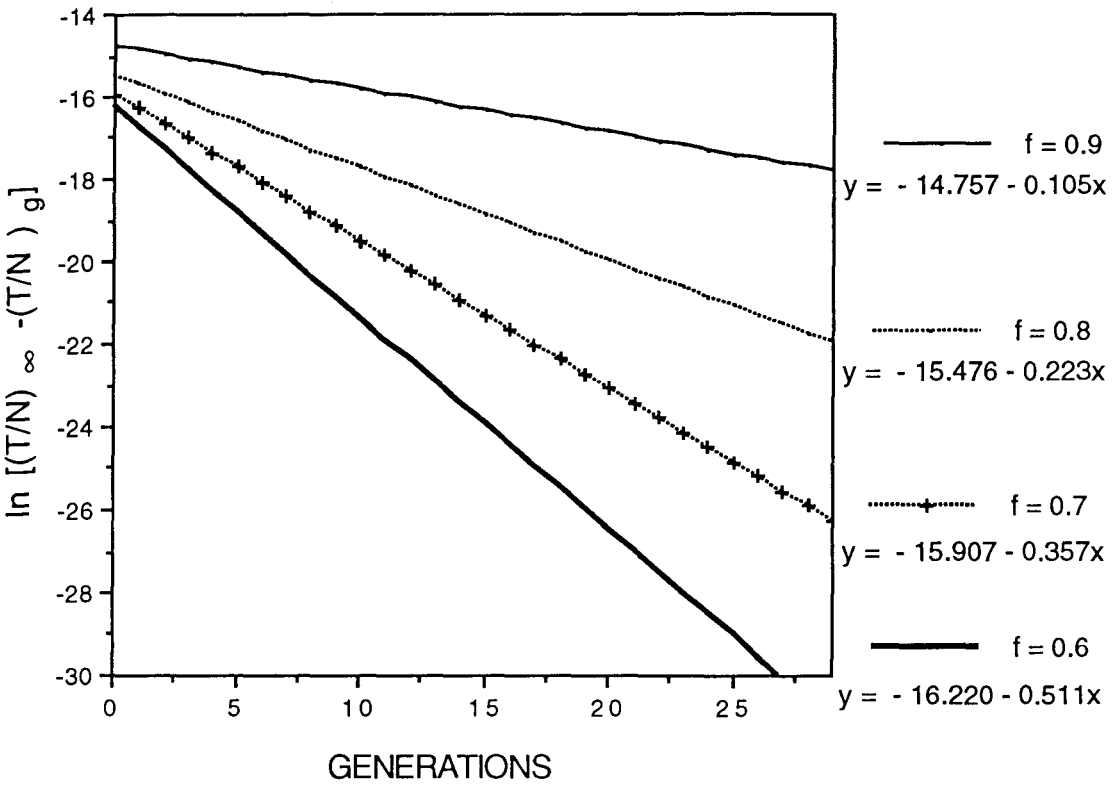


Fig. 8. Linear relationship between values of $\ln[(T/N)_\infty - (T/N)_g]$ and generations, g, for several values of the selection factor, f. Transductant-to-total ratios, $(T/N)_g$, were calculated from Equation [20] using an initial transductant-to-total ratio, $(T/N)_o$, of 2×10^{-8} and a transduction rate, $\Delta(T/N)_{trans}$, of 4×10^{-8} transductants formed per total per generation. The resulting slopes are equivalent to the natural logarithm of the selection factor, $\ln f$.

CHAPTER V

RESULTS

The Transduction System

In the transduction system, transduction was determined by quantifying the transfer of plasmid markers from a plasmid-bearing donor (RM2235) to the plasmid-free, lysogenic recipient (RM300). Transductants were identified by the possession of antibiotic markers from both plasmid Rms149 and the recipient, RM300. The absence of chromosomal markers from the donor strain, RM2235, served as a secondary confirmation.

Control experiments were performed with nonlysogenic strains to eliminate the introduction of phage to the system, thereby preventing transduction. Without the occurrence of transduction, the effects of selection alone on the transductant phenotype could be identified. In the control experiments mock-recipient (RM287) and mock-transductant (RM289) were used in place of the recipient and transductant strains. These nonlysogenic strains were otherwise identical to their lysogenic variants.

Experiments were performed at environmentally relevant concentrations (see Literature Review). Parameters including

cell concentration, generation time, and donor-to-recipient ratio, were varied to determine their effects on the rate of transduction.

Growth rates

The donor, recipient, transductant, mock-recipient and mock-transductant strains were developed as described in the Materials and Methods section. Each strain plays a specific role in the transduction model and possesses several distinctive characteristics (Table 1). In the control experiments, nonlysogenic strains were used in place of lysogenic ones to prevent the occurrence of transduction. In an effort to determine the validity of this substitution, experiments were conducted to determine how the maximal specific growth rates differ between these strains.

Batch cultures of each strain were grown in duplicate in PMM-c supplemented with 0.5% yeast extract. Cell densities were monitored over a 7 h period using a Klett-Summerson Photoelectric Colorimeter supplied with a 660 nm filter. Figures 9-11 show the changes in bacterial cell densities that were observed over time.

Maximal specific growth rates were obtained by analyzing periods of exponential increase. Growth rate, μ, was determined using the formula,

$$N/N_o = e^{\mu t} \qquad [2b]$$

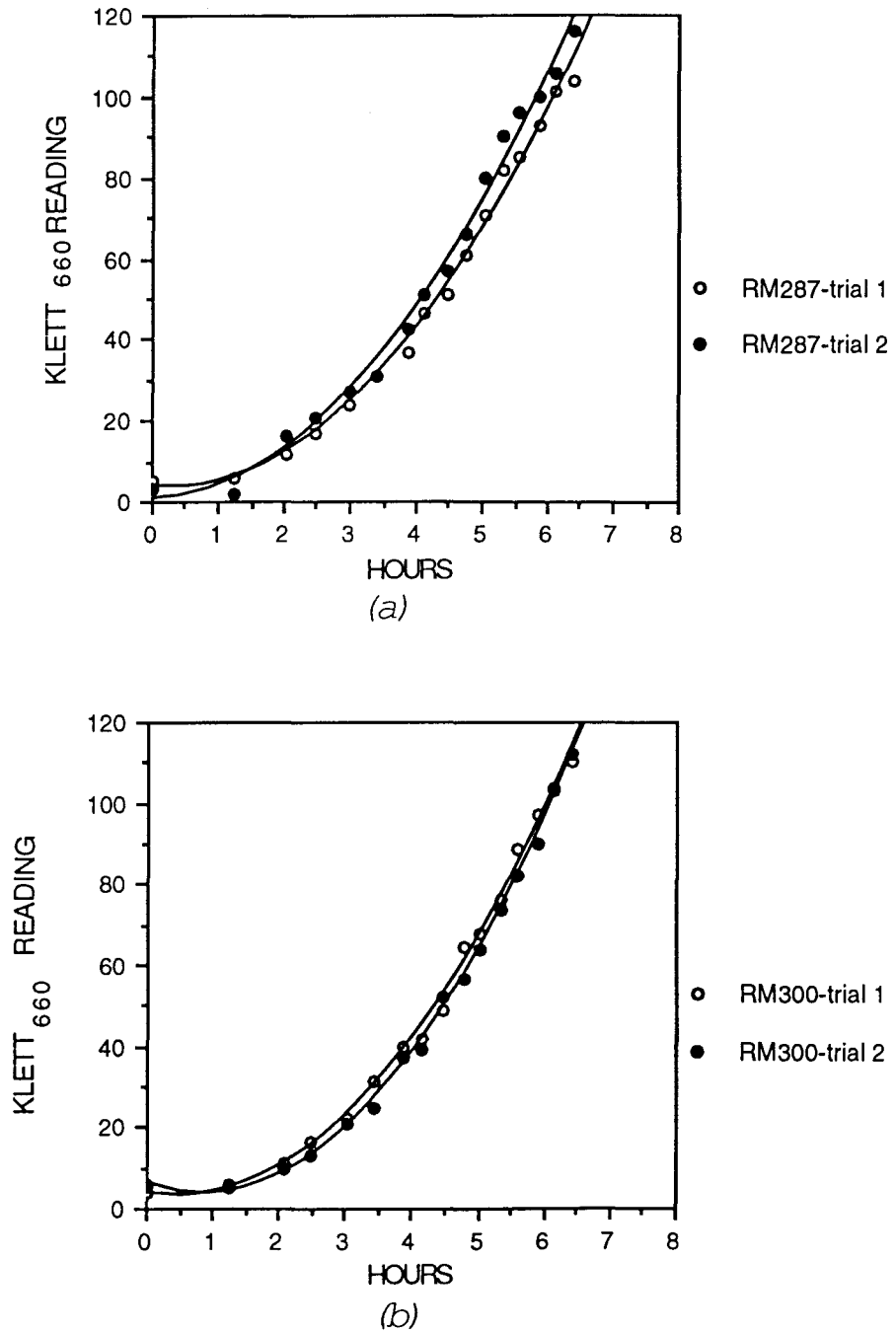


Fig. 9. Changes in *P. aeruginosa* cell density was observed over time in duplicate trials. Growth in PMM-c media supplemented with 0.5% yeast extr act was observed for the following strains: (*a*) mock-recipient, RM287 and (*b*) recipient, RM300.

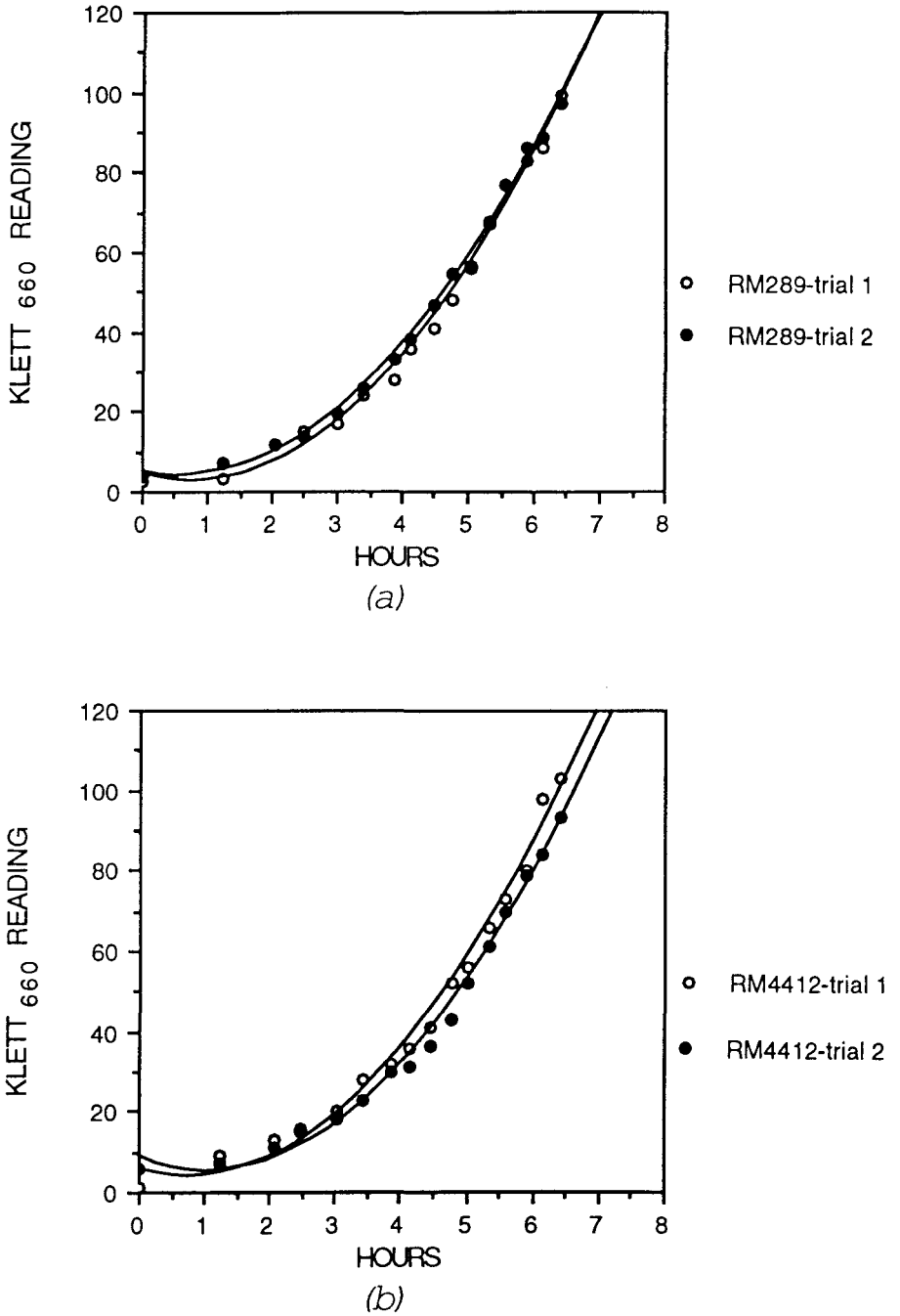


Fig. 10. Changes in *P. aeruginosa* cell density was observed over time in duplicate trials. Growth in PMM-c media supplemented with 0.5% yeast extr act was observed for the following strains: (*a*) mock-transductant, RM289 and (*b*) transductant, RM4412.

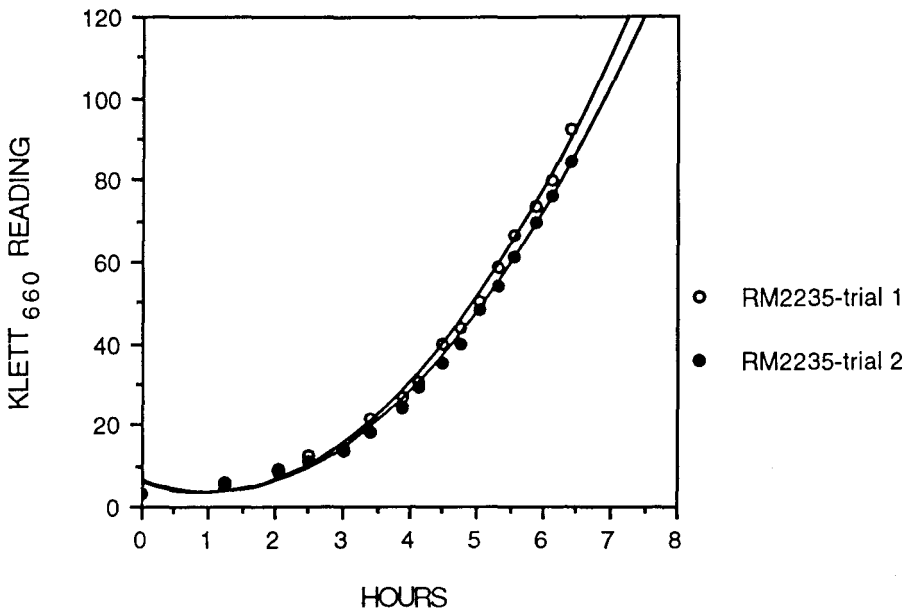


Fig. 11. Changes in *P. aeruginosa* donor strain (RM2235) density was observed over time in duplicate trials. Cells were grown in PMM-c media supplemented with 0.5% yeast extr act.

where N represents bacterial density and t is time in hours. Table 2 gives the maximal specific growth rates and the corresponding doubling times for all of the strains used in the transduction model. The plasmid-bearing donor, RM2235, was found to have the fastest rate of growth.

Interestingly, the plasmid-bearing strains, RM4412 and RM289, grew significantly faster than the otherwise identical plasmid-free strains RM300 and RM287. Previous studies on the effect of bacterial plasmids on cell growth rate show that many, but not all, plasmids cause an increase in host cell generation time, presumably due to the extra burden on the economy of the cell (12).

The nonlysogenic strains, RM289 and RM287, grew somewhat slower than their lysogenic derivatives, RM4412 and RM300. Contrasting results have been found in *E. coli* (13, 39), where lambda lysogens have been found to reproduce faster than nonlysogens. Growth differences were observed only under glucose limiting conditions, where lambda lysogens maintained a high metabolic rate, while the metabolic rate of nonlysogens declined. The increased fitness of the lysogen was found to require the lambda *rex* gene, whose function is not fully understood (39). It has been postulated that the lambda *rex* gene may cause modifications in the bacterial membrane, making the lysogen to better able to assimilate the limiting nutrients (39). The slower growth rates of F116 lysogens in this study may indicate the absence of a gene analagous to

TABLE 2

Maximal specific growth rates of *P. aeruginosa* strains measured in batch culture

Strain	Role in Model	Maximal Specific Growth Rates (h^{-1})			Doubling Time
		trial 1	trial 2	average ± std dev	(min)
RM2235	DONOR	0.5012	0.5297	0.516 ± 0.020	80.7
RM289	MOCK TRANSDUCTANT	0.5381	0.4664	0.502 ± 0.051	82.8
RM4412	TRANSDUCTANT	0.4824	0.4696	0.476 ± 0.009	87.4
RM287	MOCK RECIPIENT	0.4713	0.4338	0.453 ± 0.027	91.9
RM300	RECIPIENT	0.4368	0.4572	0.447 ± 0.014	93.1

lambda's *rex* gene, which allows increased fitness of its bacterial host under adverse environmental conditions.

Estimates for selection in the continuous culture model were made from the maximal specific growth rates observed in batch culture (Table 2). Because the donor strain had the greatest rate of growth, it may be expected to present the most competition to the transductant population. The amount of selection expected in the transduction chemostat experiments was estimated from the maximal growth rates of the transductant strain (0.476/h) and donor strain (0.516/h) in batch culture. The selection coefficient was calculated as the relative difference between the these two batch growth rates or $(\alpha-\beta)/\beta$, where α is the maximal growth rate of the transductant and β is the maximal growth rate of the donor (11, 24). Based on growth rates in batch culture, the transductant populations in chemostat experiments may expected to decrease at a rate of -0.0775 or 7.75% per h.

Selection coefficients for control chemostat experiments were estimated from differences in the growth rates of the mock-transductant (0.502/h) and the donor (0.516/h) strains in batch culture. Decrease in the relative concentration of mock-transductants was estimated at -0.0271 or 2.71% per h, suggesting that there would be greater selection against the transductant population in the transduction experiments than against the mock-transductants in the control experiments.

The mathematical model presented in the preceding chapter expresses changes in transductant frequency as the result of both selection and transduction. The effect of selection alone in the transduction experiments is estimated from selection of the mock-transductant in control experiments, where transduction cannot occur. However, the transductant strain was found to grow somewhat slower than the its nonlysogenic derivative in batch culture (Table 2). Therefore selection experienced by the transductants in the transduction experiments may be greater than selection experienced by the mock-transductants in the control experiments. Thus, the contribution of selection in transduction experiments may be underestimated. In the mathematical model, an underestimation of selection would lead to an underestimation of transduction as well. However, this situation is preferred, since it insures that elevated levels of transductants in the transduction experiments may be attributed to the occurrence of transduction, and not to increased fitness of the transductant strain.

Determination of transducing particle-to-total phage ratio

The production of transducing particles is a relatively infrequent event. With concentrations of transducing particles as low as 0.01/ml, it was not practical to assay for their concentrations directly from the chemostat. Therefore, experiments were performed to determine the frequency at which

transduction particles are produced, so that their concentrations could be estimated from titers of total bacteriophage. In the transduction system, where the donor is the plasmid-bearing parent, only the lysis of donors will result in the production of plasmid-containing transducing particles.

As it was possible that transducing-particle frequency might depend on the ratio of plasmid-bearing donors and lysogenic recipients present, an experiment was designed to determine whether altering the donor-to-recipient ratio would affect the proportion of transducing particles produced in the phage-particle population. Five batch cultures were inoculated with varying amounts of donor and recipient bacteria to yield donor-to-recipient ratios of 42:1 to 1:244. The cultures were assayed for the number of plasmid-bearing transductants they could produce. Single measurements of transducing particle (P) and bacteriophage F116 virion (ϕ) concentrations, determined at 16 h and 24 h, are presented in Table 3.

It was found that the density of phage increased with the donor-to-recipient ratio. However, the ratio of plasmid-containing transducing particles per phage remained fairly constant over the range of donor-to-recipient ratios explored. These observations indicate that phage particles were produced predominantly from the lysis of plasmid-bearing donor cells.

TABLE 3

Concentrations of transducing particle and phage F116, and their ratios observed in batch culture experiments

Trial	Incubation Time (h)	Donor: Recipient[a]	transd particle density[b] (P)	phage density (ϕ)	transd particle: phage ratio (P/ϕ)
1	25	42.0	4.4×10^{3}	4.0×10^{10}	1.1×10^{-7}
2	16	5.0	7.0×10^{3}	1.0×10^{10}	7.0×10^{-7}
	25	3.1	4.8×10^{3}	2.5×10^{10}	1.9×10^{-7}
3	16	0.10	5.6×10^{3}	6.8×10^{9}	8.3×10^{-7}
	25	0.17	2.3×10^{3}	9.1×10^{9}	2.5×10^{-7}
4	16	0.024	1.2×10^{3}	2.0×10^{9}	6.0×10^{-7}
	25	0.012	4.8×10^{2}	1.1×10^{9}	4.4×10^{-7}
5	16	0.010	2.0×10^{1}	7.0×10^{7}	2.9×10^{-7}
	25	0.0041	5.1×10^{0}	2.3×10^{7}	2.2×10^{-7}

[a] At total cell concentrations of 10^{8}-10^{9} cells per ml.

[b] Values for transducing particles obtained by selecting for plasmid bearing transductants on rifampicin and carbenicillin selection plates.

The average value for the transducing-particles-to-total-phage ratio was $4.0 \pm 2.5 \times 10^{-7} P/\phi$. This value was used to estimate the densities of plasmid-containing transducing particles in the chemostat experiments .

Evaluation of media for transductant recovery

Experiments were performed to determine whether the choice of primary selective agent (*i.e.*, antibiotics) had an effect on the efficiency of transductant recovery. The recipient strain, RM300, was transduced using a cell-free F116 lysate prepared on the plasmid-bearing strain, RM2235. The transduction mixture was divided and spread on onto plates containing selective media, one for each different antibiotic combination. Transduction of the plasmid was ascertained by the transfer of the carbenicillin or streptomycin resistance marker. Recovery on rifampicin was also examined because of its use as a counterselection against donors in the transduction system.

Table 4 gives the apparent transduction frequencies for each of the antibiotic selection media tested. Transduction frequencies are reported as the number of transductants per plaque forming unit (PFU). Selection on carbenicillin allowed for the greatest recovery of transductants. Streptomycin selection resulted in up to 1370-fold lower apparent transduction frequencies. This observation may be due to differences in bacterial survival resulting from the

TABLE 4

Efficiency of various primary selective media for the recovery of transductants of RM300 with plasmid Rms149

	Number of transductants/10^6 PFU			
MOI	Cb Sm Rf[a]	Sm Rf	Cb Rf	Cb
100	0.0005	0.0006	0.34	0.42
10	0.0006	< 0.0006[b]	0.82	1.5
1.0	0.006	< 0.006[b]	0.25	3.9
0.1	< 0.06[b]	< 0.06[b]	6.1	9.3
0.01	< 0.6[b]	< 0.6[b]	14.8	8.0

[a]The abbreviations Cb, Sm, and Rf refer to the antibiotics carbenicillin, streptomycin, and rifampicin respectively.

[b]These values indicate experimental detection limits for the MOIs indicated.

different inhibitory actions of the two antibiotics. Streptomycin blocks the polymerization of amino acids in protein synthesis, whereas carbenicillin interferes with cell wall synthesis (16). Therefore, transductants retrieved on carbenicillin selective medium have a greater opportunity to express their resistance, thus increasing their frequency of survival. All transductants retrieved on carbenicillin selection plates were found to be resistant to streptomycin as well. Because carbenicillin allowed for the highest recovery of transductants, it was chosen as the primary selection marker for enumerating transductants in the chemostat experiments.

Media selection

The chemostat experiments were performed in PMM-c medium supplemented with yeast extract as the sole nutritional source. In preparation for the chemostat studies, preliminary experiments were performed in batch culture to determine the cell densities that could be supported by a range of yeast extract concentrations. Growth tubes of minimal media were prepared using yeast extract concentrations ranging from 10^{-1} to 10^{-9}% (w/v). Ten microliters of exponential-phase recipient (RM300) were inoculated into each tube to give 10^2, 10^3, or 10^4 cells/ml, using less cells for lower dilutions of yeast extract. The cultures were enumerated after two days by plate counts.

Yeast extract concentrations and the recipient cell densities that resulted are shown in Figure 12. These initial batch experiments aided in determining the concentrations of yeast extract needed to obtain specific cell densities in continuous culture.

The Control Experiments : Determining the selection coefficient

To quantify the effects of selection alone on the transductant phenotype, control experiments were performed using nonlysogenic strains. Because no source of phage was added to the system, transduction could not occur. The control experiments were performed as outlined in Figure 13. Inoculum from mock-transductant (RM289), donor (RM2235) and mock-recipient (RM287) cultures were added to the chemostat vessel containing PMM-c supplemented with 4 x 10^{-5}% yeast extract. Yeast extract supplied at this concentration was found to support 10^5 to 10^6 CFU/ml (Figure 12). The mock-transductants were introduced as a minority population, $<10^{-3}$% of the total cell concentration, to mimic an emerging transductant population. After inoculation, the pump was activated to begin continuous flow at the selected dilution rate. The reservoir was sampled at least once daily as described in the Materials and Methods section. The samples were assayed for concentrations of donors, mock-recipients, and mock-transductants.

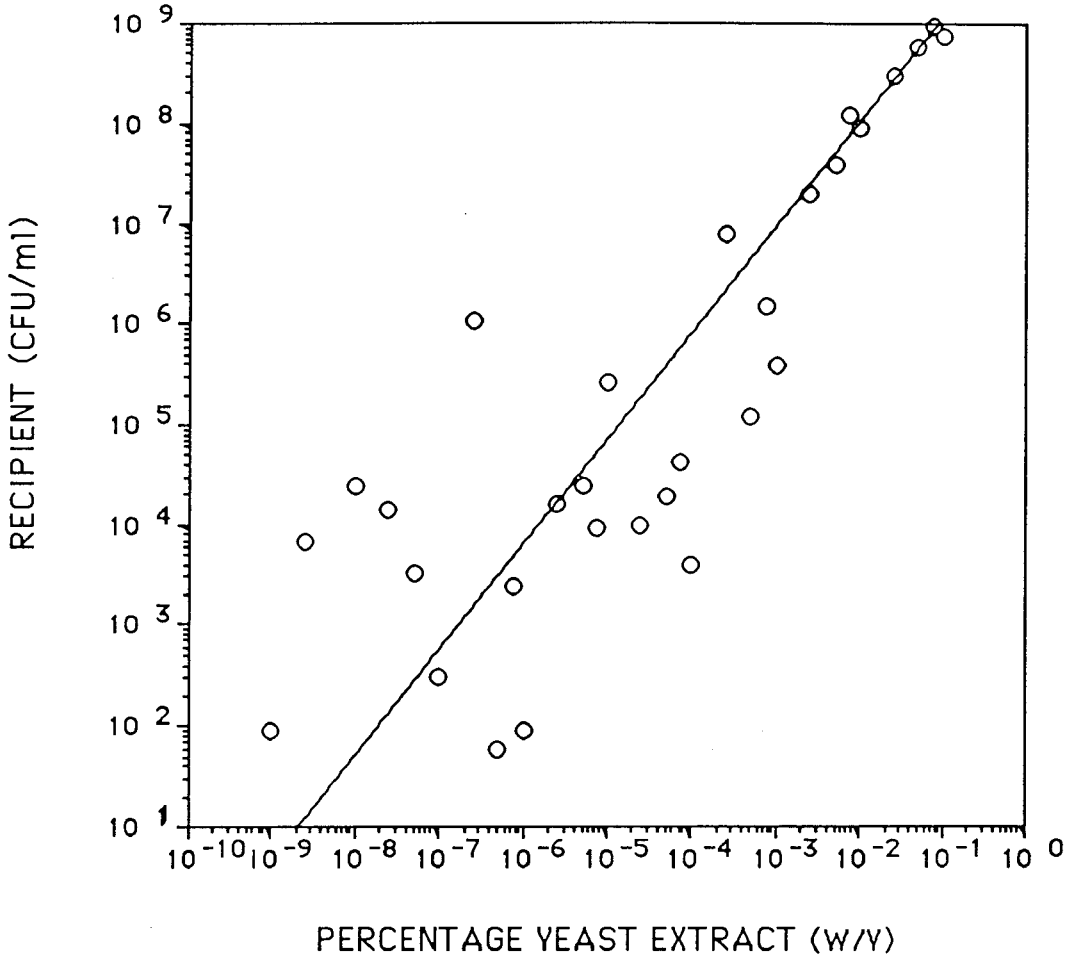


Fig. 12. Cell densities supported when yeast extract is provided as the sole nutritional source. Colony forming units of the recipient strain (RM300) observed after 2 days of incubation on PMM-c media supplemented with indicated concentrations of yeast extract.

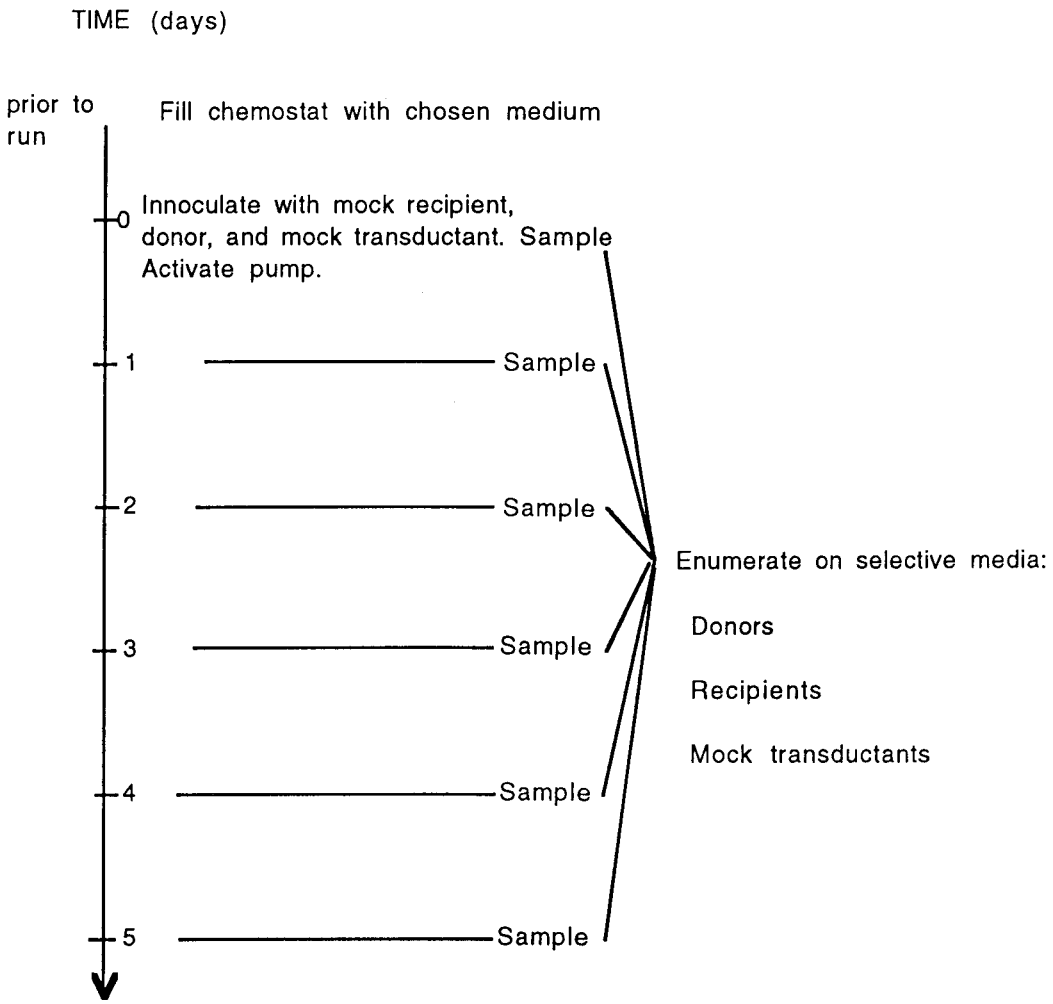


Fig. 13. Experimental protocol for control experiments in the chemostat.

Control experiments were performed at cell generation times ranging from 1.9 to 13.4 h. Experiments were evaluated to determine how the relative number of mock-transductants changed with time. Over the range of generation times tested, the number of mock-transductants per total was found to decrease exponentially with time. For each experiment, the selection coefficient, s, was solved by inputting the ratio of mock-transductants per total bacteria, T_t/N_t, observed at time-points, t, into the equation:

$$(T_t/N_t) = (T_o/N_o)\ e^{st} \qquad [17]$$

Values were calculated for selection per generation and selection per hour (Table 5). All values for the selection coefficient were negative, indicating selection against the mock-transductant. Figures 14-16 show the changes in the relative concentration of mock-transductants observed over generations in continuous culture.

The rate of selection per hour was not dependent on generation time. Selection per hour had an average value of -0.013 ± 0.006, or 1.3% selection per hour, comparable to the value of 2.7% selection per hour estimated from growth rates in batch culture (Table 2).

However, there was a significant increase in selection per generation as the length of generation time increased (Figure 17). Dykhuizen (11) observed a similar phenomena in

TABLE 5

Calculated selection coefficients, *s*, computed from observed changes in mock-transductants concentration with respect to the total population[a]

Expt	Generation Time (h)	*s* (± s.e.) (gen^{-1})	*s* (± s.e.) ($hour^{-1}$)
1	1.9	-0.021 ± 0.011	-0.011 ± 0.006
2	2.4	-0.028 ± 0.019	-0.012 ± 0.008
3	8.3	-0.041 ± 0.033	-0.005 ± 0.004
4	10.1	-0.140 ± 0.030	-0.014 ± 0.003
5	13.4	-0.282 ± 0.134	-0.021 ± 0.010

[a]Selection coefficients and standard errors were obtained for each experiment by solving s in Equation [17] using the nonlinear modeling function of SYSTAT.

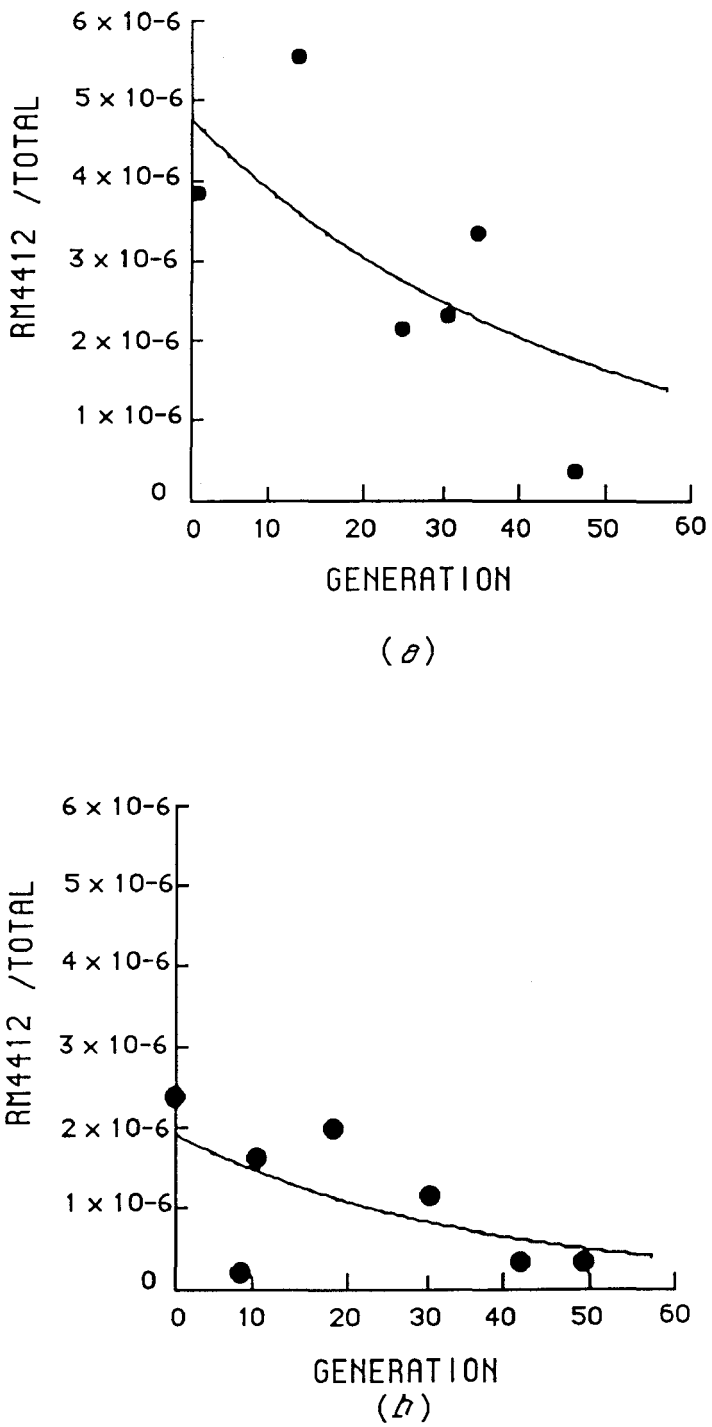
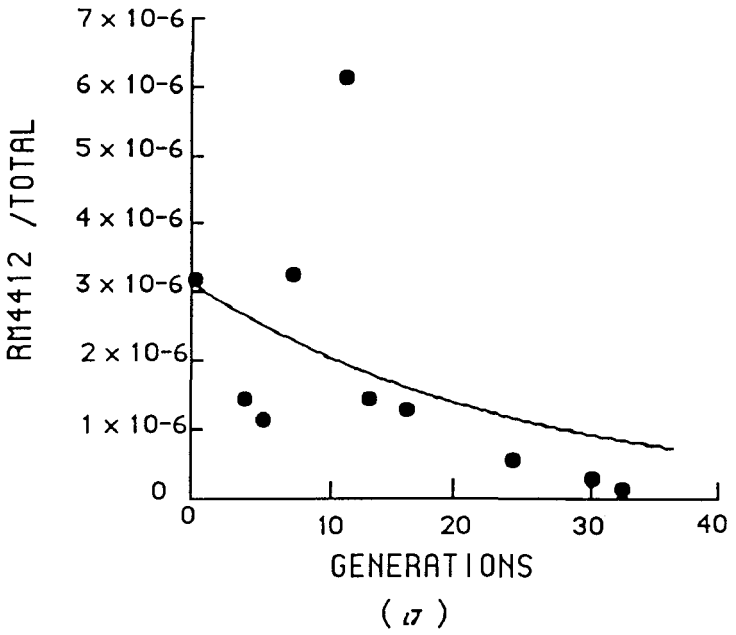


Fig. 14. Changes in the ratio of mock-transductants per total bacteria in control experiments: (*a*) Experiment 1, performed at generation time of 1.9 h and (*b*) Experiment 2, performed at generation time of 2.4 h



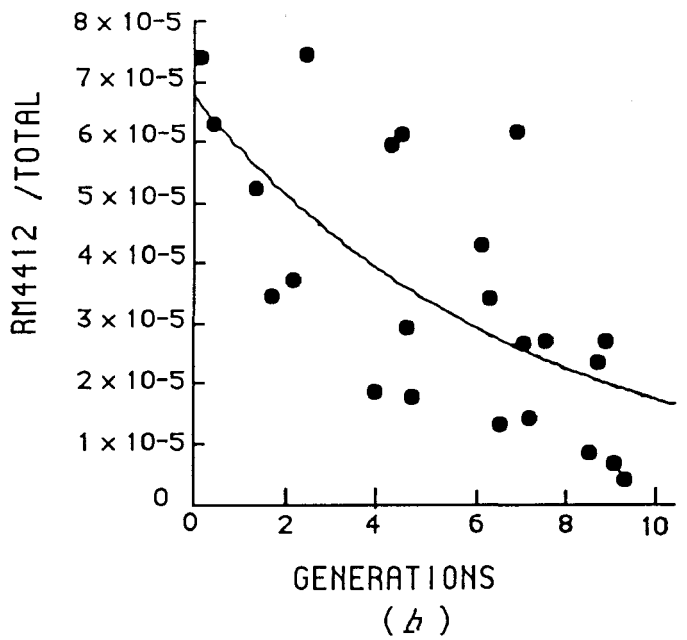


Fig. 15 Changes in the ratio of mock-transductants per total bacteria in control experiments: (*a*) Experiment 3, performed at generation time of 8.3 h and (*b*) Experiment 4, performed at generation time of 10.1 h

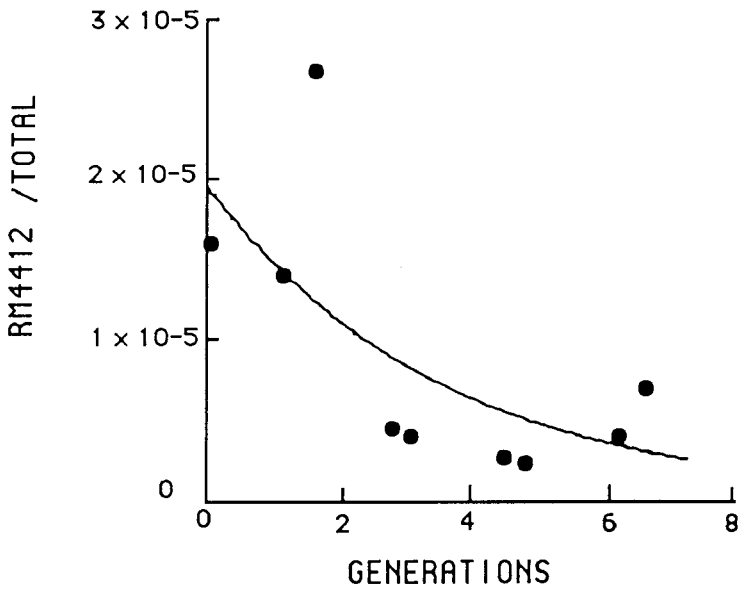


Fig. 16. Changes in the ratio of mock-transductants per total bacteria in control experiment 5, performed at generation time of 13.4 h

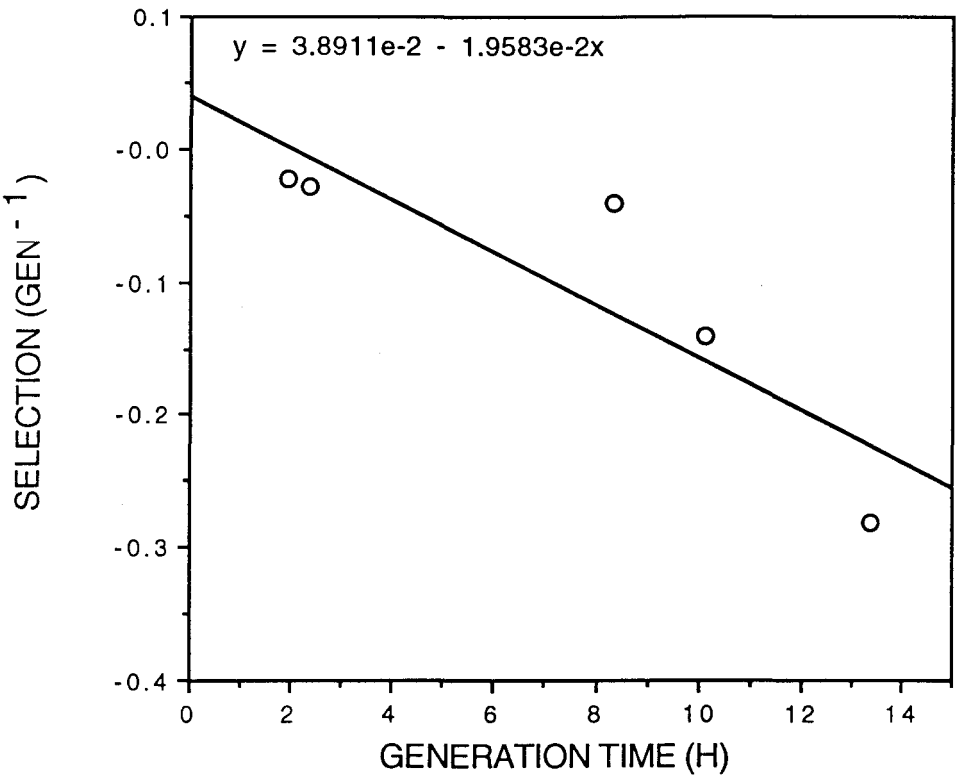


Fig. 17. Selection per generation against the mock-transductant decreased as the length of cell generation time increased in control experiments.

populations of tryptophan auxotrophs and wild-type *E. coli*. He found that selection increased with generation time for generation times up to 3 h. At generation times greater than 3 h, he found that selection dropped to zero, indicating no selection against the auxotroph (11). In this study, selection against the mock-transductant was found to increase with generation time over the range of generation times tested (1.7-13.4 h).

Initial Transduction Trials

Initial experiments were performed to determine whether it was possible to observe transduction in continuous culture. A system to study transduction was designed. The chemostat was first inoculated with the recipient, RM300, and allowed to achieve a stable concentration. After 60 to 120 hours, the donor strain, RM2235, was inoculated into the chemostat reservoir.

Figures 18 and 19 show the results of these initial transduction trials. When the donor strain was added to an established population of recipients, it experienced a dramatic drop in cell number. In these trials, the recipient population had an opportunity to become adjusted to chemostat growth conditions, before the inoculation of the donor. The dramatic decreases in the donor cell number may reflect a disadvantage due to the donor's physiological state. In one case, the donor population was able to recover from this

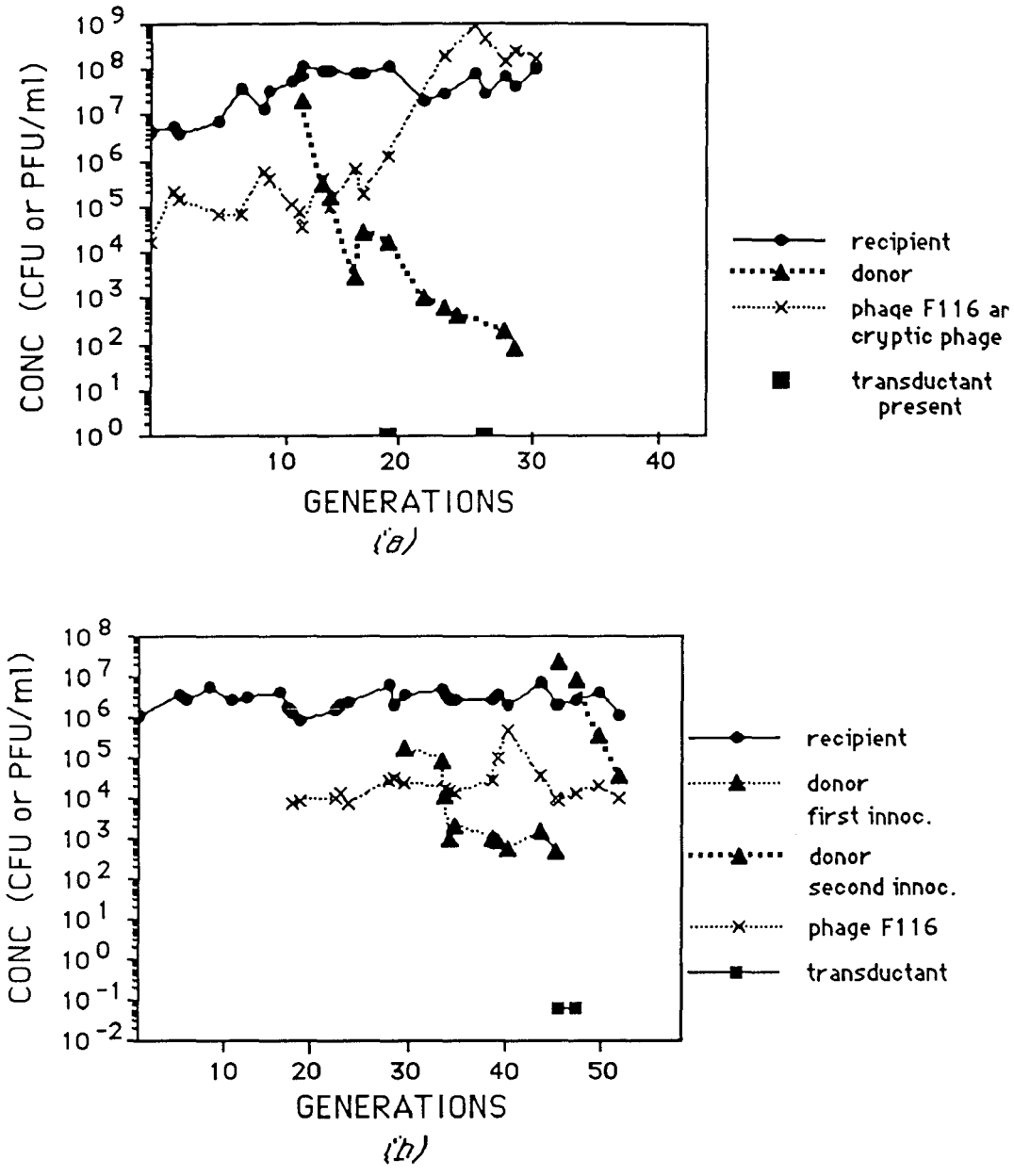


Fig. 18. Initial transduction trials showing sporadic appearance of transductants. Delayed inoculation results in a decline of donor population: (*a*) experiment performed at generation time of 10.2 h and (*b*) experiment performed at 4.3 h generation time. Donor strain was inoculated twice.

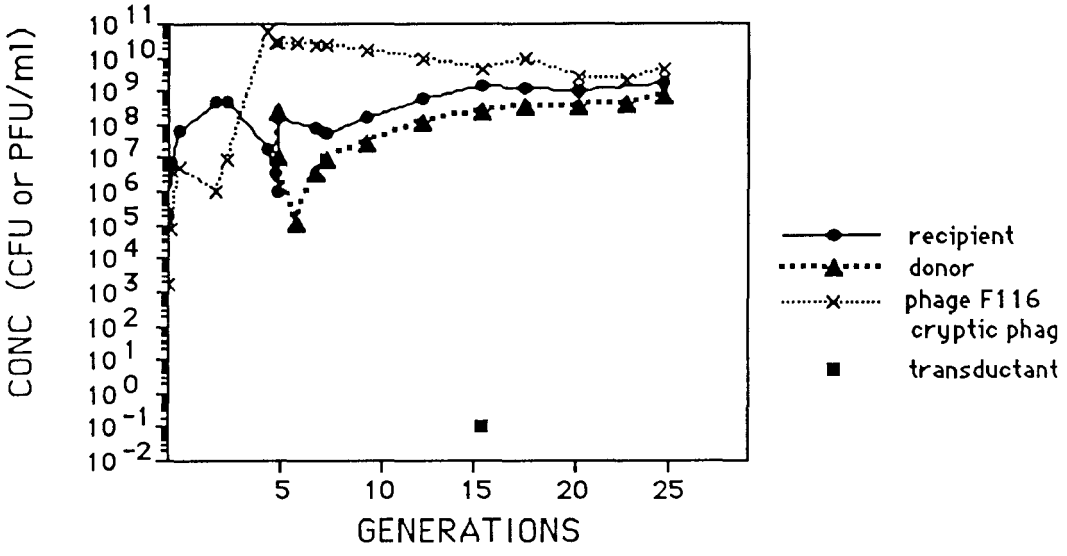


Fig. 19. Initial transduction experiment performed at 9.7 h generation time. Decline and subsequent recovery of donor strain.

initial population loss (Figure 19). In this experiment, the donor was inoculated at 50 h, whereas in the other initial trials, donors were introduced after 120 h (Figure 18). This difference in the time of inoculation may have allowed the donor to effectively compete with the recipient strain. In all three experiments, transductants were observed sporadically just at the level of detection. With only the occasional appearance of transductants, it was impossible to determine whether the transductant population was increasing, decreasing, or stabilizing.

Transduction Experiments

The transduction protocol was modified to overcome the difficulties encountered in the initial transduction experiments. The final transduction protocol is outlined in Figure 20. The donor and recipient strains were inoculated at the same time into the chemostat reservoir and allowed to grow in batch. This gave an opportunity for transductants to accumulate, so that changes in the transductant densities could be traced. After one to two days in batch, the pump was activated to begin continuous culture at the selected dilution rate. The reservoir was sampled at least once daily. The samples were assayed for concentrations of donors, plasmid-bearers, recipients, transductants and bacteriophages as described in the Materials and Methods section.

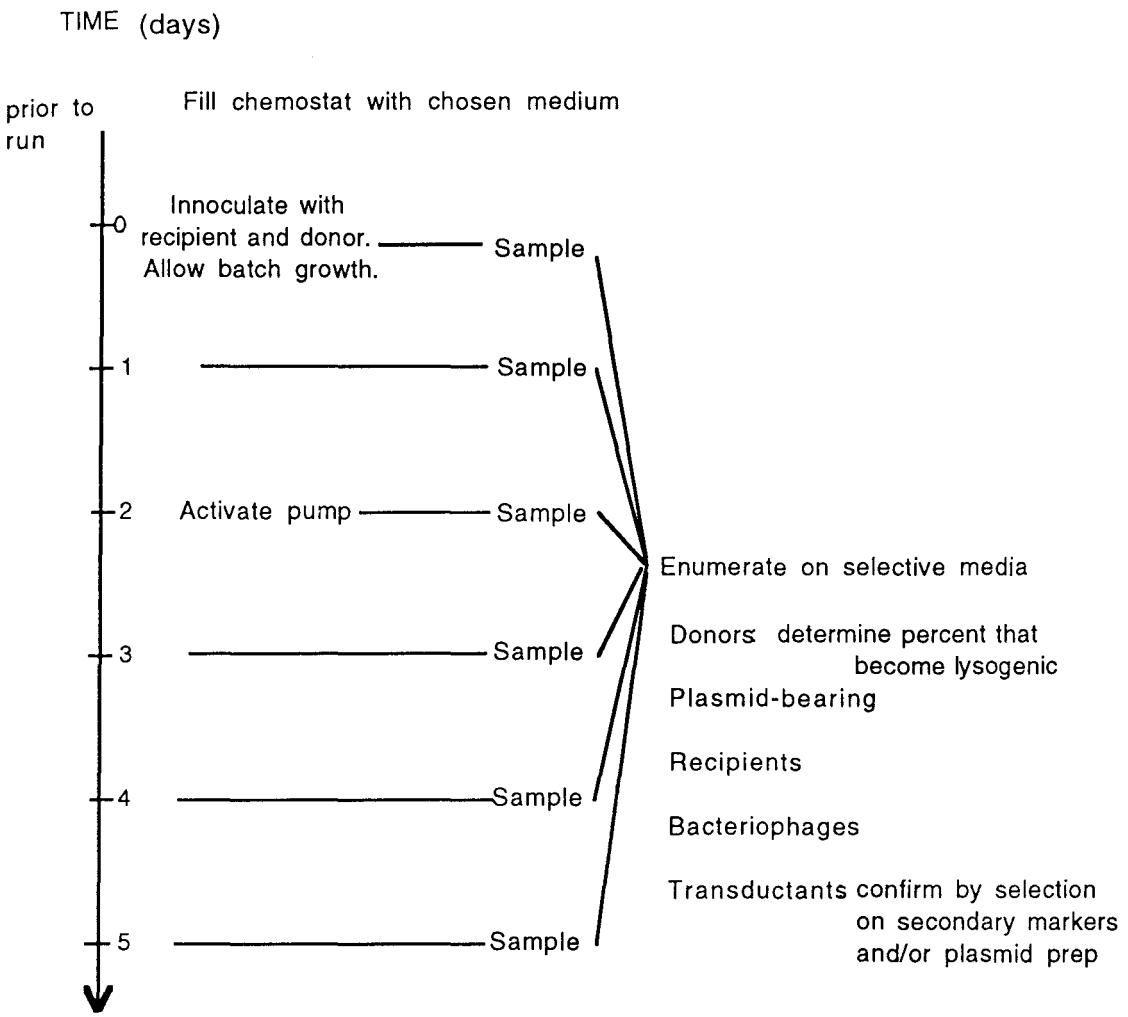


Fig. 20. Experimental protocol for transduction experiments in the chemostat.

Donors were identified by their expression of the chromosomal gene for nalidixic acid resistance. To assess the stability of plasmid Rms149 in the transduction experiments, cells possessing plasmid-encoded resistance to carbenicillin were also enumerated. Plasmid-bearing cell concentrations did not vary significantly from donor concentrations, indicating stable maintenance of plasmid Rms149 by the bacteria. A representative experiment is shown in Figure 21.

Putative transductants were tested for the presence of genetic markers from the recipient chromosome and plasmid Rms149, and the absence of markers from the donor chromosome. Selected transductants were verified by performing plasmid minipreparations. Plasmid DNA was digested with *Eco*R1 and analyzed by agarose gel electrophoresis (Figure 22). Plasmid Rms149 was found in all transductant isolates tested.

Transduction experiments were performed over a range of cell concentrations, cell generation times, and donor-to-recipient ratios. The transduction experiments are described in Table 6, where they are grouped according to their cell densities and phage-to-bacteria ratios (PBR).

Figures 23 and 24 show experiments 8 and 9, performed at high cell concentrations (>10^8 CFU/ml). Figures 25-33 show experiments 10-18, which were performed at lower cell concentrations (10^5 to 10^7 CFU/ml), low phage-to-bacteria ratios (< 1), and cell generation times ranging from 1.7 to

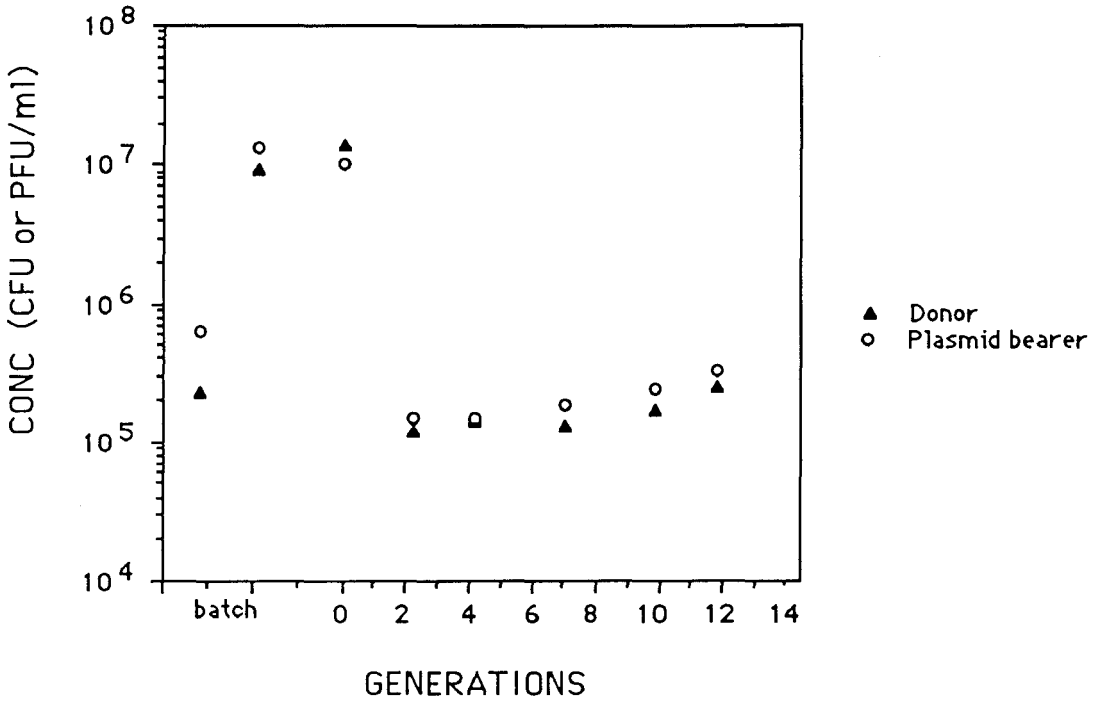


Fig. 21. Plasmid-bearing cell concentrations did not vary significantly from donor concentrations. A representative experiment is presented (Experiment 19). Donor and plasmid-bearing bacteria concentrations were enumerated on L-agar supplemented with nalidixic acid and carbenicillin, respectively.

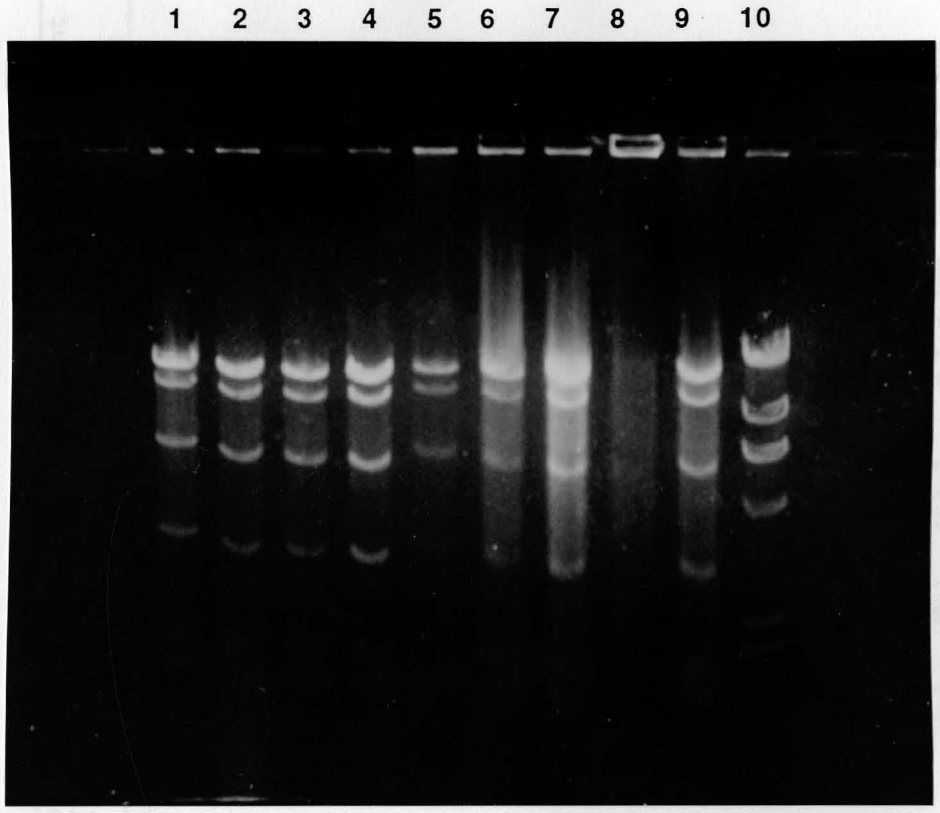


Fig. 22. Restriction patterns of plasmid DNA isolated from transductants. Lanes 1-6, *Eco*R1 digests of transductants from experiments 15, 18, 17, 12, 16, and 14, respectively; lane 7-9 *Eco*R1 digests of RM2235 (donor), RM300 (recipient), and RM289 (mock-transductant); lane 10 *Hin*dIII digests of lambda DNA.

TABLE 6

Transduction Experiments: A summary of conditions and equilibrium concentrations

	Expt	Yeast Extract Conc	Gen. Time (h)	Total Cells (ml-1)	PBR	Donor Cells (ml-1)	Recipient cells (ml-1)	Phage F116 (ml-1)
High Cell Conc.								
	8	5.0×10^{-1}	2.8	7.3×10^{8}	0.01	2.8×10^{8}	4.5×10^{8}	1.1×10^{7}
	9	1.6×10^{-1}	9.8	6.4×10^{8}	1.66	9.7×10^{7}	6.4×10^{8}	8.3×10^{8}
Low Cell Conc. Low PBR[a]								
	10	4.0×10^{-5}	2.1	3.8×10^{5}	0.25	5.7×10^{4}	3.2×10^{5}	2.7×10^{4}
	11	4.0×10^{-5}	3.0	1.6×10^{6}	0.15	2.7×10^{5}	1.3×10^{6}	7.6×10^{4}
	12	4.0×10^{-5}	3.4	4.6×10^{5}	0.09	1.1×10^{5}	3.5×10^{5}	9.8×10^{4}
	13	4.0×10^{-5}	6.9	3.8×10^{6}	0.22	6.1×10^{5}	3.2×10^{6}	5.9×10^{5}
	14	4.0×10^{-5}	8.4	2.7×10^{6}	0.14	1.6×10^{5}	2.5×10^{6}	5.9×10^{5}
	15	4.0×10^{-5}	9.2	3.2×10^{6}	0.12	6.6×10^{5}	2.6×10^{6}	2.1×10^{5}
	16	4.0×10^{-5}	10.3	1.1×10^{7}	0.03	7.3×10^{5}	1.1×10^{7}	2.1×10^{5}
	17	4.0×10^{-5}	13.6	1.4×10^{7}	0.02	2.8×10^{6}	1.1×10^{7}	1.7×10^{5}
Low Cell Conc High PBR								
	18	9.0×10^{-9}	8.9	7.5×10^{5}	3.60	5.2×10^{5}	2.3×10^{5}	6.5×10^{5}
	19	4.0×10^{-5}	9.6	2.5×10^{6}	125.0	2.5×10^{6}	8.0×10^{4}	1.2×10^{7}
	20	4.0×10^{-5}	7.8	4.0×10^{5}	66.0	3.3×10^{5}	6.3×10^{4}	6.2×10^{6}

[a] Phage-to-bacteria ratio

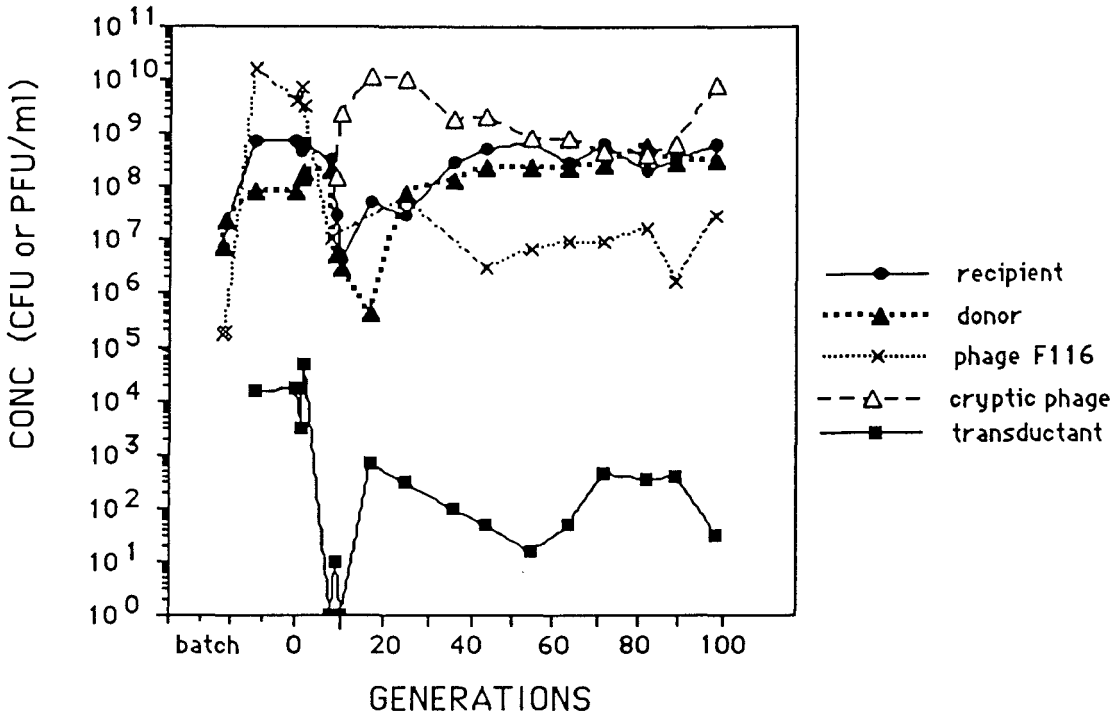


Fig. 23. Experiment 8. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. A cryptic phage was induced. Experiment 8 was performed at 5 x 10^{-1}% yeast extract, a donor-to-recipient ratio of 0.11 and a 2.8 h generation time.

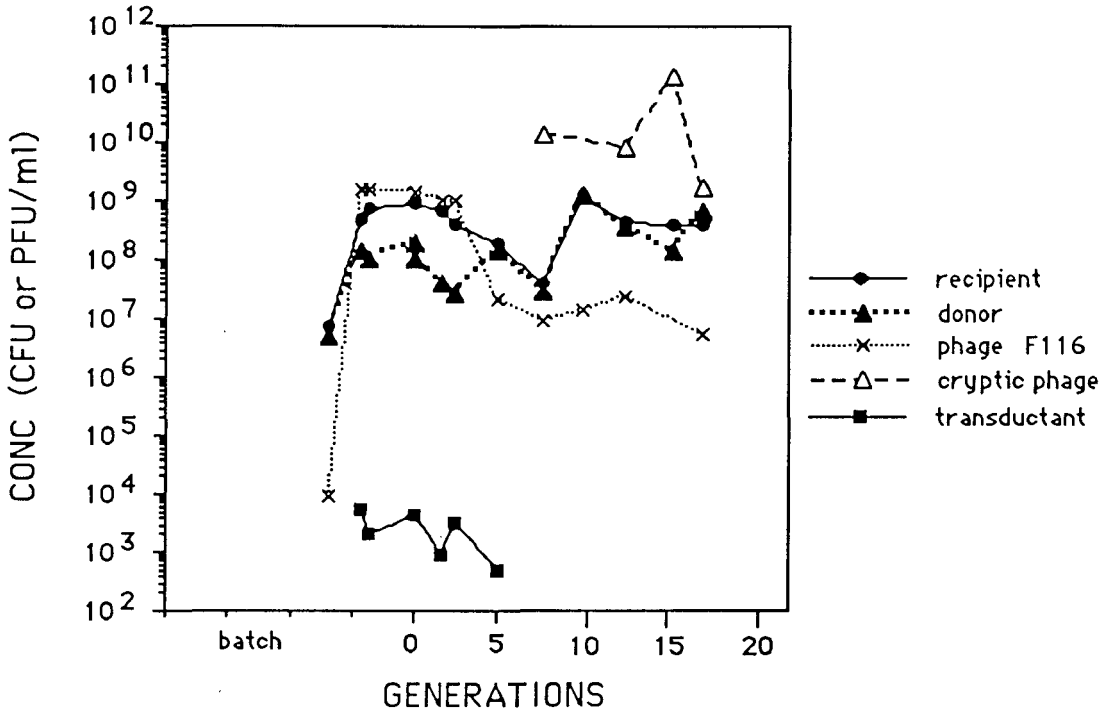


Fig. 24. Experiment 9. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. A cryptic phage was induced. Experiment 9 was performed at 1.6 x 10^{-1}% yeast extract, a donor-to-recipient ratio of 0.54 and a 9.8 h generation time.

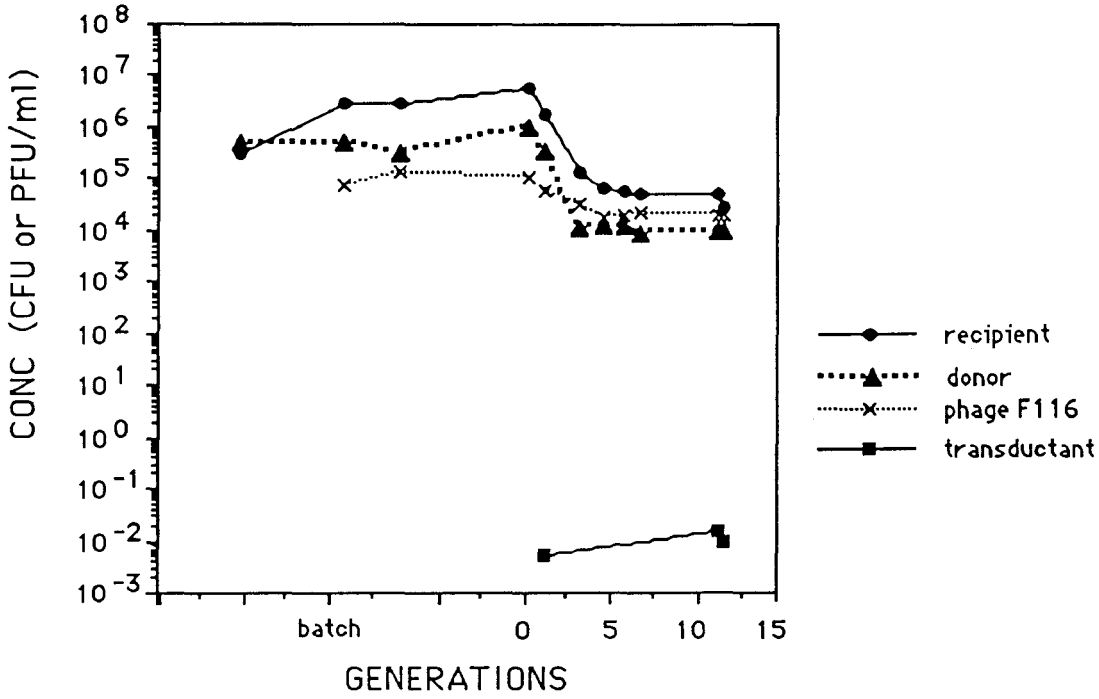


Fig. 25. Experiment 10. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 10 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.17 and a 2.1 h generation time.

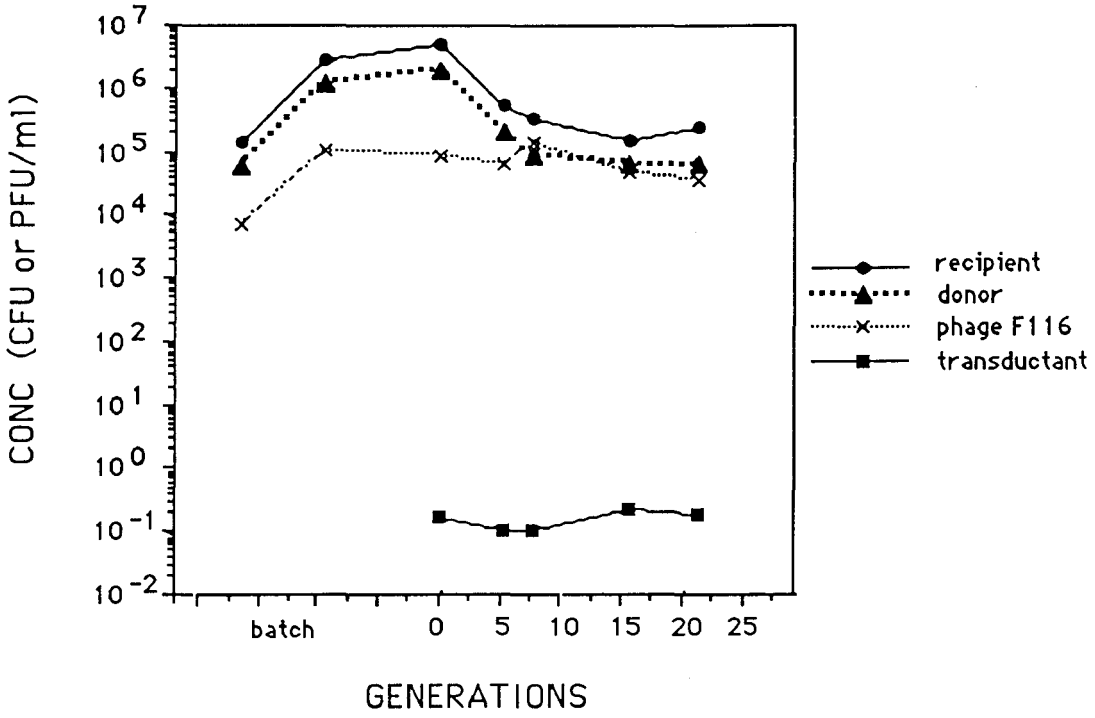


Fig. 26. Experiment 11. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 11 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.32 and a 3.0 h generation time.

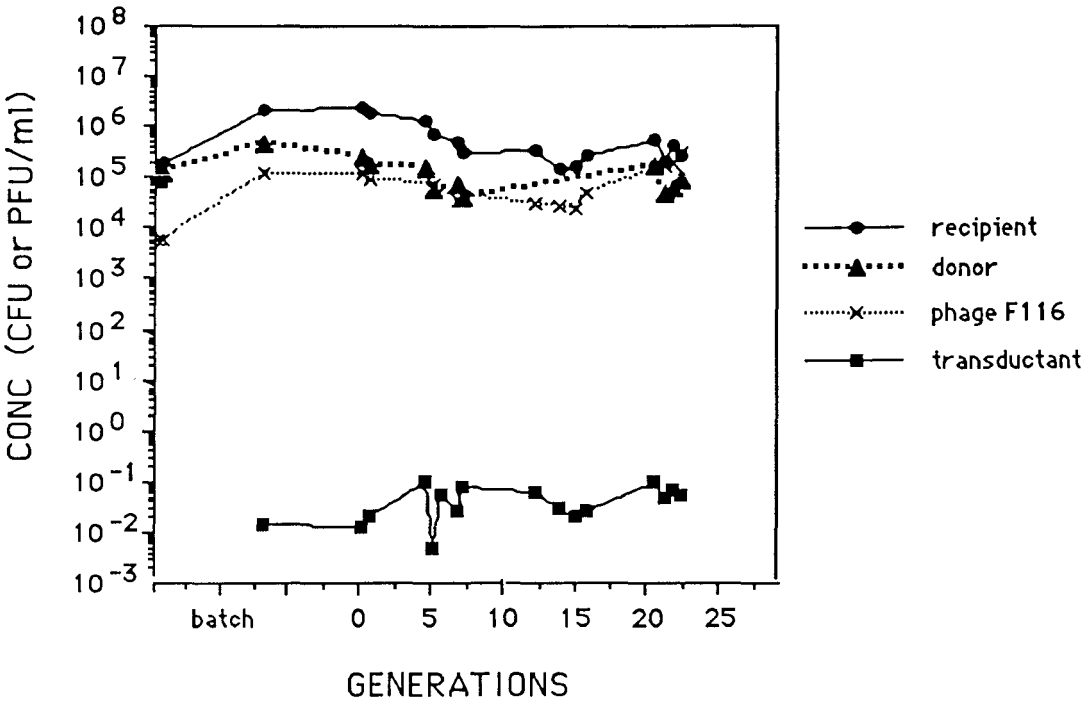


Fig. 27. Experiment 12. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 12 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.14 and a 3.4 h generation time.

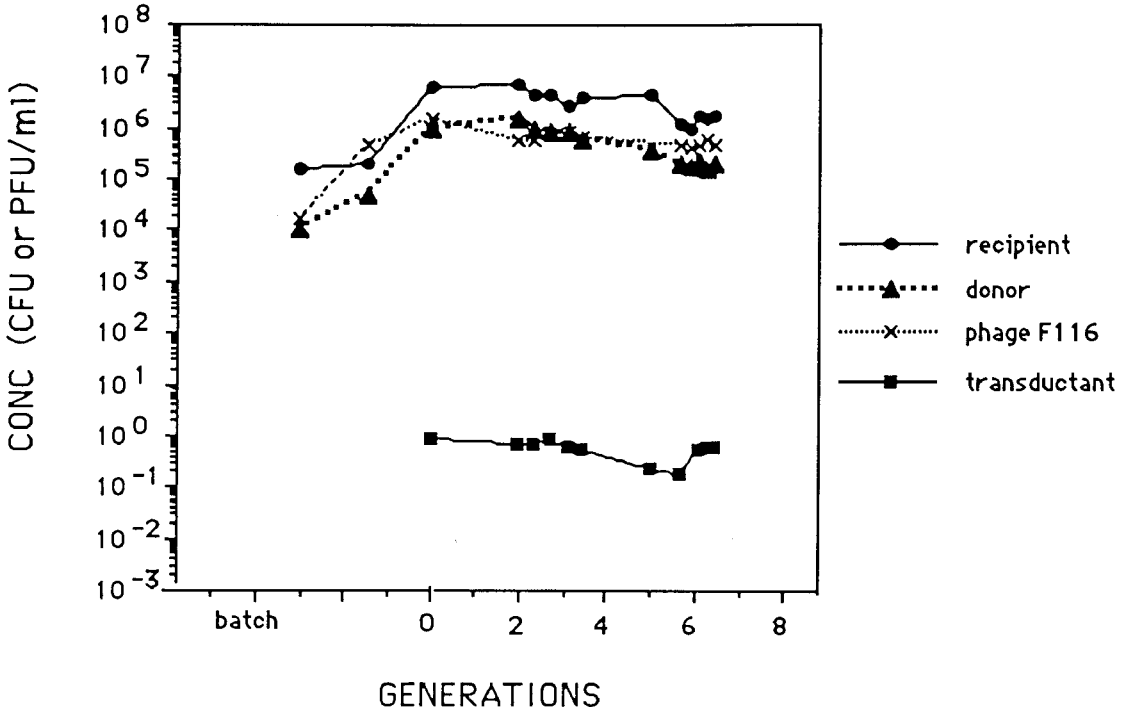


Fig. 28. Experiment 13. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 13 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.15 and a 6.9 h generation time.

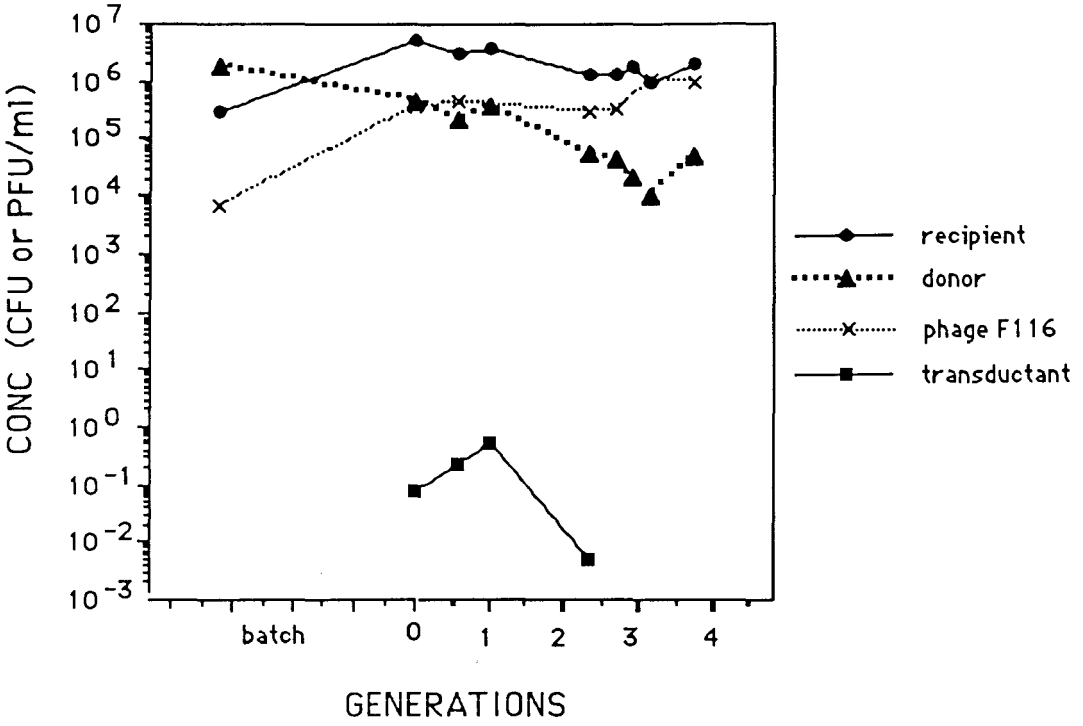


Fig. 29. Experiment 14. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 14 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.08 and a 8.4 h generation time.

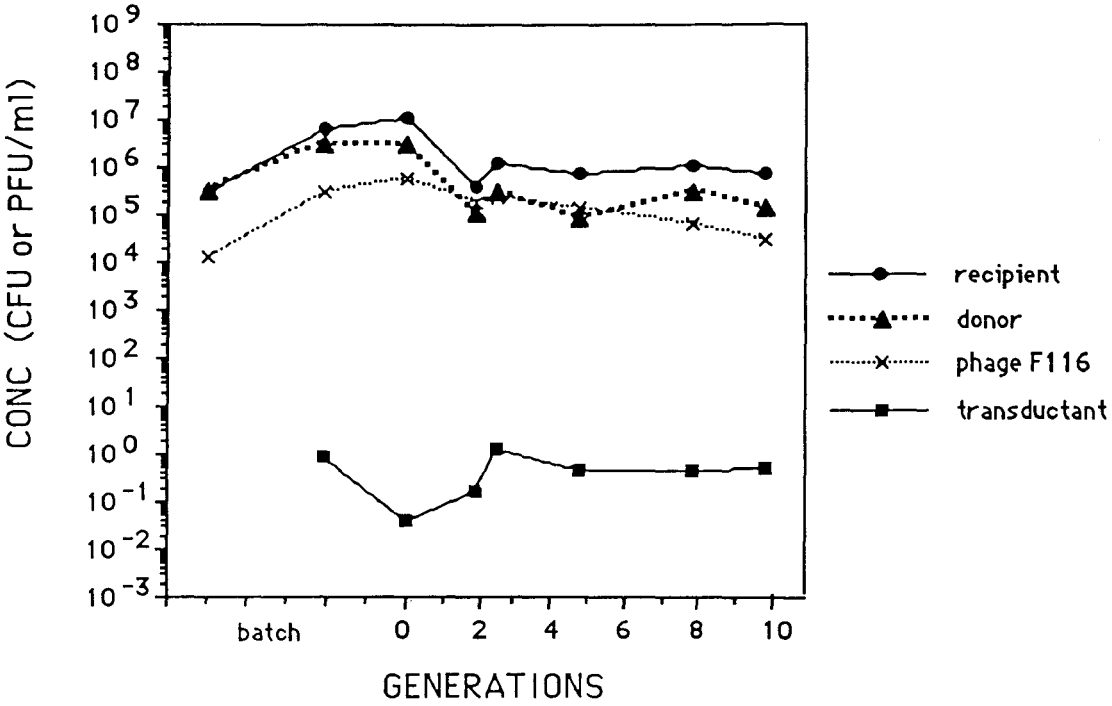


Fig. 30. Experiment 15. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 15 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.20 and a 9.2 h generation time.

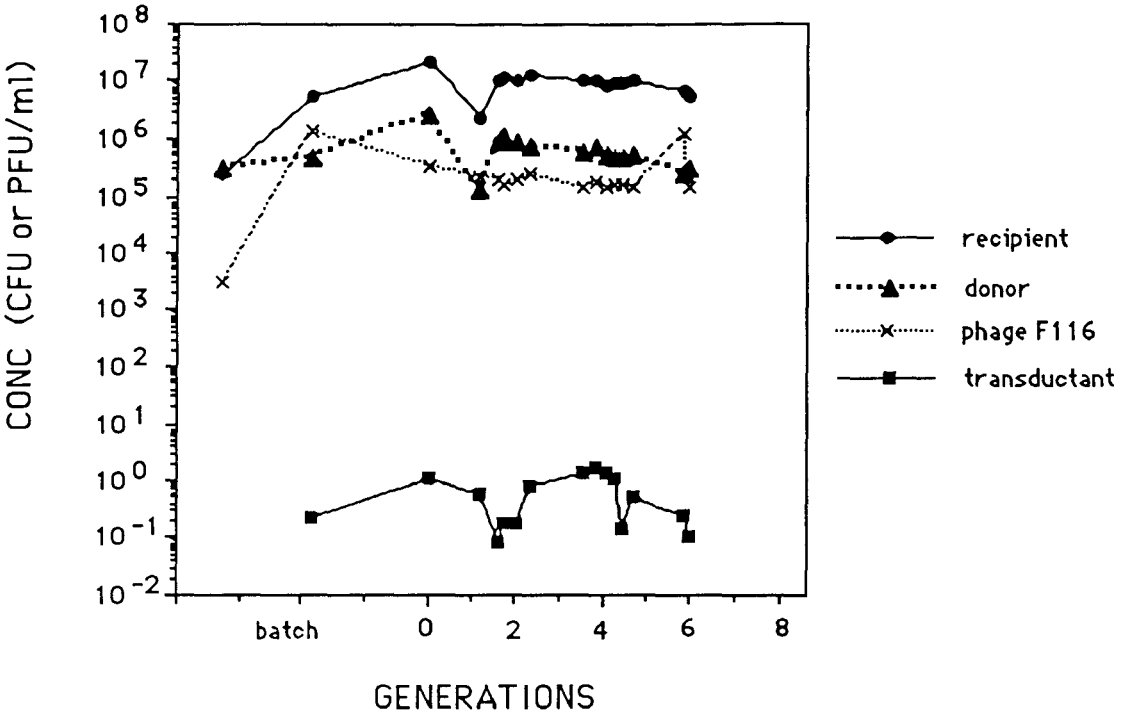


Fig. 31. Experiment 16. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 16 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.06 and a 10.3 h generation time.

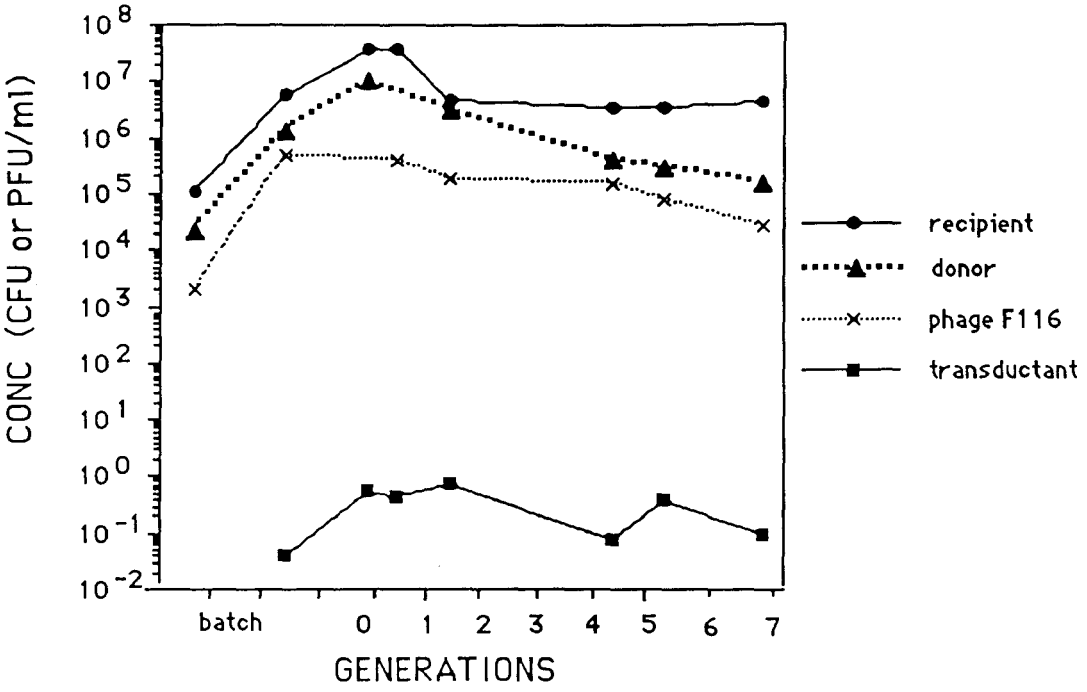


Fig. 32. Experiment 17. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 17 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 0.09 and a 13.6 h generation time.

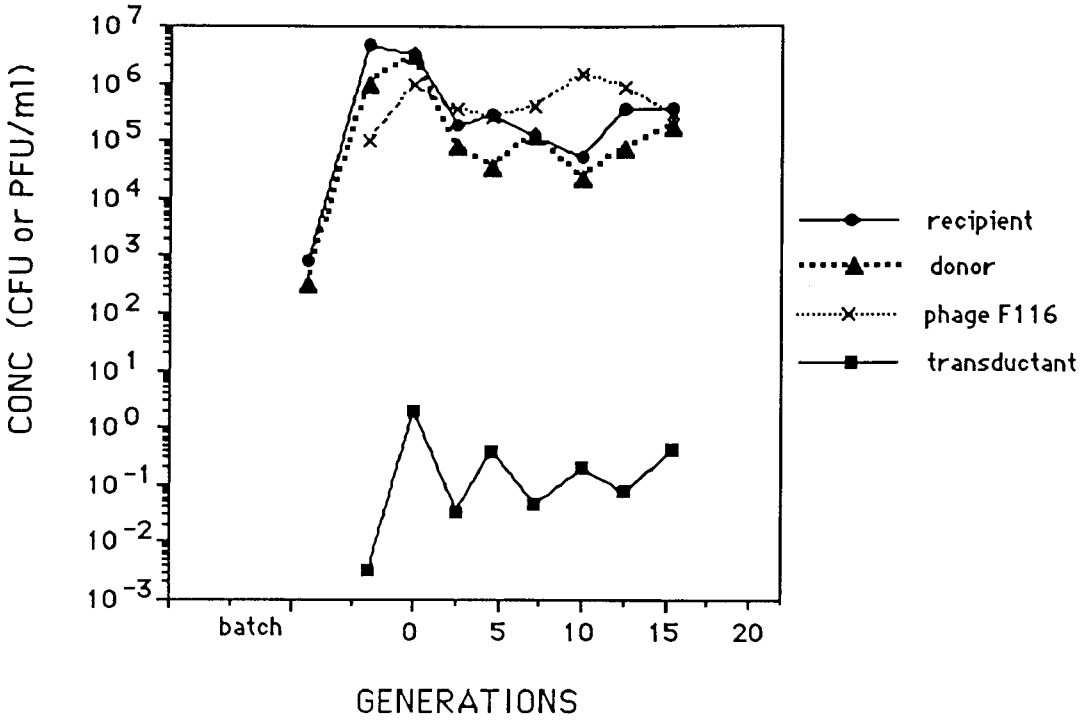


Fig. 33. Experiment 18. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 18 was performed at 9.0 x 10^{-9}% yeast extract, a donor-to-recipient ratio of 0.33 and a 8.9 h generation time.

13.4 h. Figures 34 and 35 show the results of experiments 19 and 20, performed at low cell concentrations (10^5 to 10^7 CFU/ml) and high phage-to-bacteria ratios (> 1).

Attempts were made to obtain stable growth at cell densities of less than 10^5 CFU/ml by reducing or eliminating the yeast extract and decreasing the strength of the minimal medium. However, it was found that the *Pseudomonas* would either stabilize at approximately 10^6 cells/ml or wash out of the system.

An unusual bacteriophage was found to appear unexpectedly during the high cell concentration trials only (Figures 23-24). It was induced after 40-90 hours of continuous culture growth, and resulted in sudden increases in viral concentration. The plaques were different from F116 in that they were clearer, smaller and maintained at 1000-fold higher densities. This phage was also found in control experiments performed at 0.16% and 0.5% yeast extract, indicating that they were not simply result of an F116 mutation. Additionally, the phage appeared when the parental type strain PAO1 was grown alone in continuous culture. The phage was able to form plaques on the strain from which it originated. Phage lysates prepared from plaques were found to be unstable and decrease rapidly in the number of plaque-forming units over time. Growth of the bacterial strains in batch culture or at low cell densities had not revealed the presence of this virus.

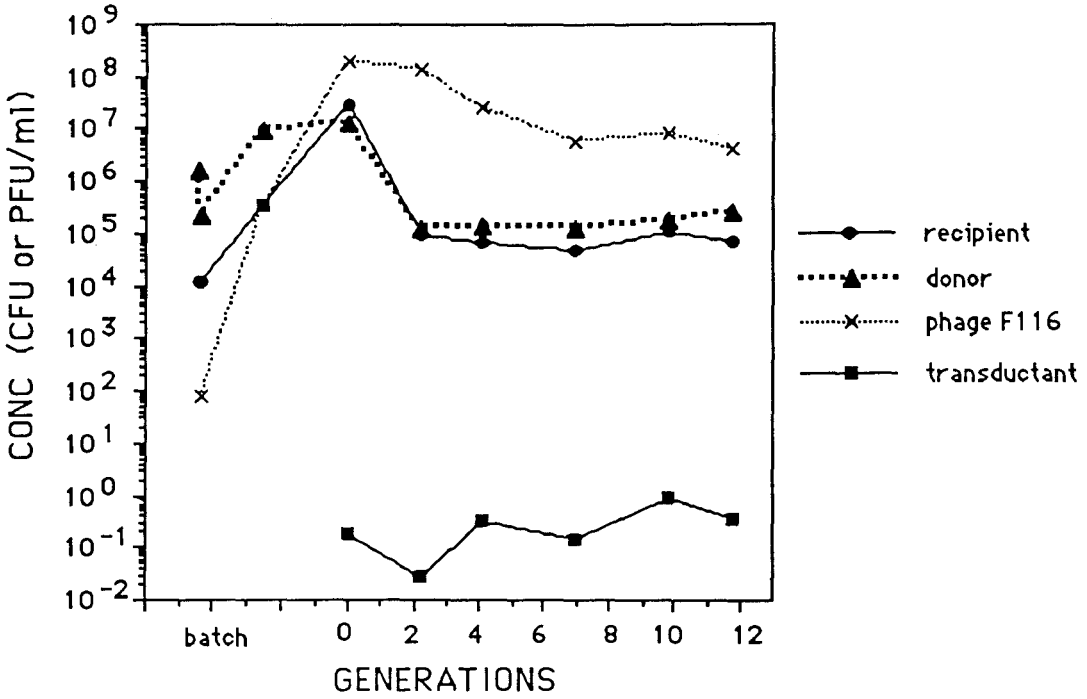


Fig. 34. Experiment 19. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 19 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 1.27 and a 9.6 h generation time.

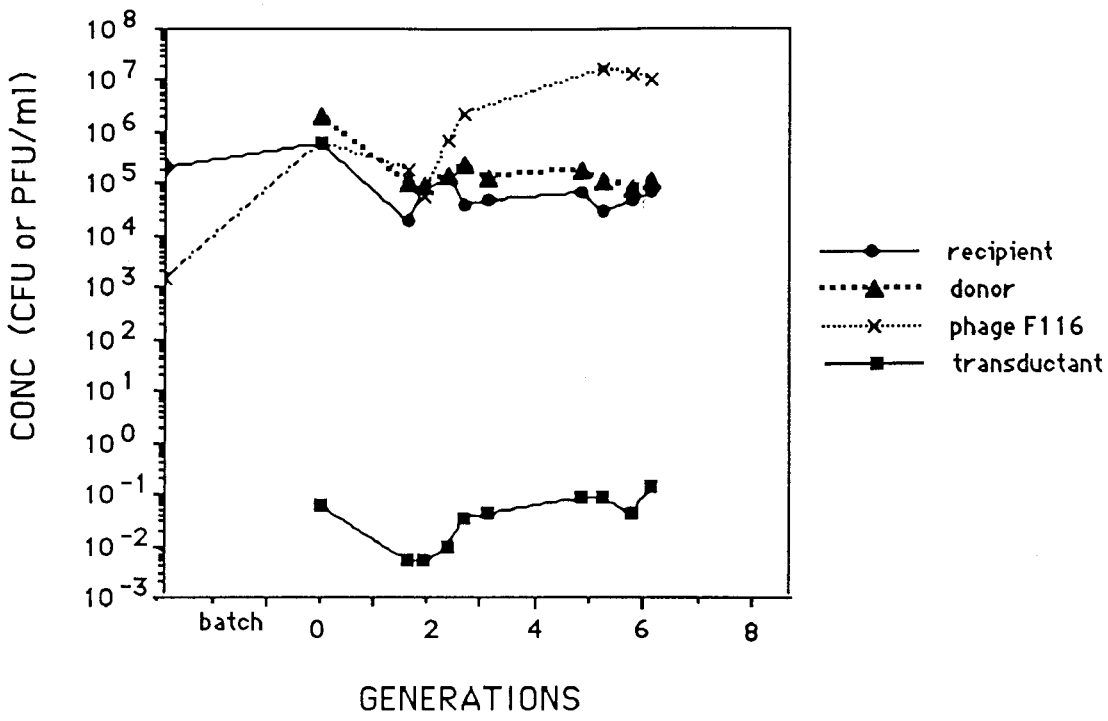


Fig. 35. Experiment 20. Concentrations of donor, recipient, transductant, and bacteriophage observed during the length of the experiment. Experiment 20 was performed at 4.0 x 10^{-5}% yeast extract, a donor-to-recipient ratio of 2.00 and a 7.8 h generation time.

Many commonly used bacterial strains are known to harbor cryptic viruses, viruses not readily detectable from the continuous release of plaque-forming virus particles (9). The induction of a cryptic virus from strain *P. aeruginosa* PAO1 was previously reported by Terry and Mattingly (62) and Terry *et al.* (63) in studies on mucoid conversion during continuous culture growth. They found high titers of phage within 30 h of incubation using 0.002 M to 0.01 M phosphorylcholine chloride or 5% glycerol as carbon sources. This virus was also able to plaque on the strain from which it was induced (62, 63).

Because the presence of the cryptic virus caused a dramatic drop in bacterial densities in the high concentration experiments, transductants were analyzed before this concentration dropped, or after transductant population recovery. Bacteriophage F116 were present at a much lower concentrations than the cryptic phage and consequently could not be enumerated by plaque assays on *P. aeruginosa* PAO1 when the cryptic phage was present. After cryptic phage induction, F116 were enumerated on the strain *P. aeruginosa* PAT1, which was resistant to cryptic phage attack. This may be due to the ability of *P. aeruginosa* PAT1 restriction endonucleases to digest cryptic phage, but not F116 DNA.

Lysogeny in Donors

Isolates of donors were examined to determine what

portion became lysogenic for F116 (Table 7). In all experiments some portion of the donors became lysogenic, although there was no discernible pattern to lysogeny establishment. In Experiment 19, where the phage-to-bacteria ratio (PBR) was 125, the donors slowly became lysogenic and remained that way. The opposite situation occurred in Experiment 17, where the PBR was 0.02. Here the majority of donors became lysogenic immediately and then nonlysogens dominated. In many of the experiments, the proportion of lysogens tended to fluctuate. Establishment of lysogeny in the donors was rarely complete, with some portion of the donors remaining nonlysogenic. This may be due to the growth advantage found in the nonlysogenic strains (Table 2).

Applying the Mathematical Model

Data obtained from the transduction experiments were analyzed to determine how well the derived model could describe changes observed in the transductant populations. Transductant-to-total ratios observed over the course of each experiment are shown in Figures 36-42, with each point representing a single measurement. In all runs, the transductant-to-total ratio was found to increase overall. In some experiments, the increase followed a specific pattern (expts 10-12, 14, 16, 20), but in other experiments, the path appeared more random (expts 8-9, 13, 18).

TABLE 7

Fraction of donors exhibiting lysogeny for bacteriophage F116 in Experiments 11-20

Expt 11 (D:R[a] .32)		Expt 12 (D:R .14)		Expt 13 (D:R .15)		Expt 14 (D:R .08)		Expt 15 (D:R .20)		Expt 16 (D:R .06)		Expt 17 (D:R .09)		Expt 18 (D:R .33)		Expt 19 (D:R 1.27)		Expt 20 (D:R 2.00)	
Hrs[b]	fract lysog[c]	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog	Hrs	fract lysog
0	.25	0	.04	21	.96	0	<.12[d]	0	.12	49	>.96	0	>.96	0	<.04	0	<.04	0	<.04
20	.38	1	.25	34	.83	22	.58	30	.38	61	.96	22	>.96	32	.88	18	.67	22	.08
50	.38	25	.04	36	.54	27	.63	50	.20	67	>.96	42	>.96	55	.75	44	>.96	35	.08
66	.50	48	.21	39	.54	30	.63	68	.38	70	>.96	49	>.96	77	.25	65	>.96	38	.25
74	.12	50	.17	42	.46	42	.88	73	.25	73	>.96	62	.43	96	.38	83	>.96	41	.29
98	.38	64	.29	44	.46			94	.25	86	.96	101	<.04	118	.38	110	.79	43	.08
116	.12	66	.06	55	.75			122	>.88	89	.88	113	<.04	142	.50	138	>.96	60	.83
		72	.44	60	.32			140	.62	91	.96	136	.12	166	>.96	157	>.96	63	.88
		73	.29	61	.37									190	.50			68	.96
		90	.80	62	.48													70	.83
		102	.61	64	.42														
		118	.17	65	.35														
		120	.46																
		122	.83																
		124	.65																

[a] Donor-to-recipient ratio

[b] Total number of hours in chemostat

[c] Fraction of donors tested that exhibited spontaneous induction of phage F116

[d] Values given as < or > denote the limit of detection

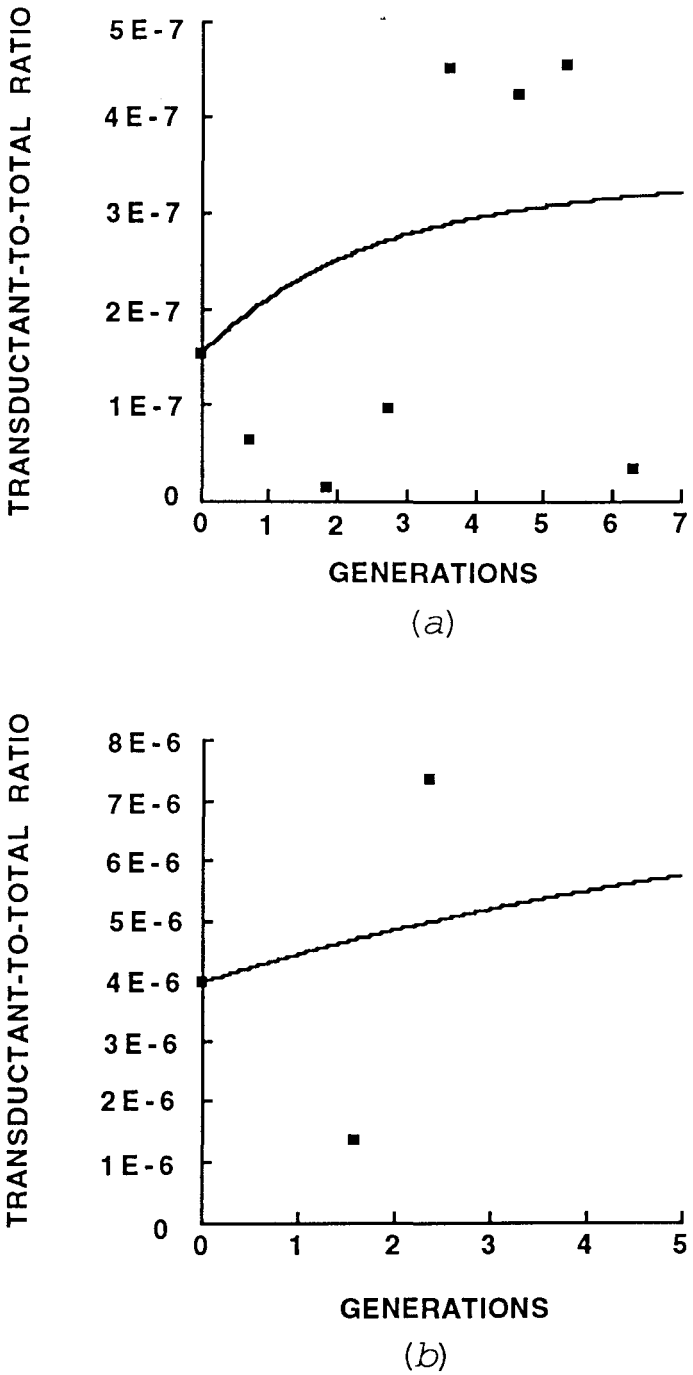
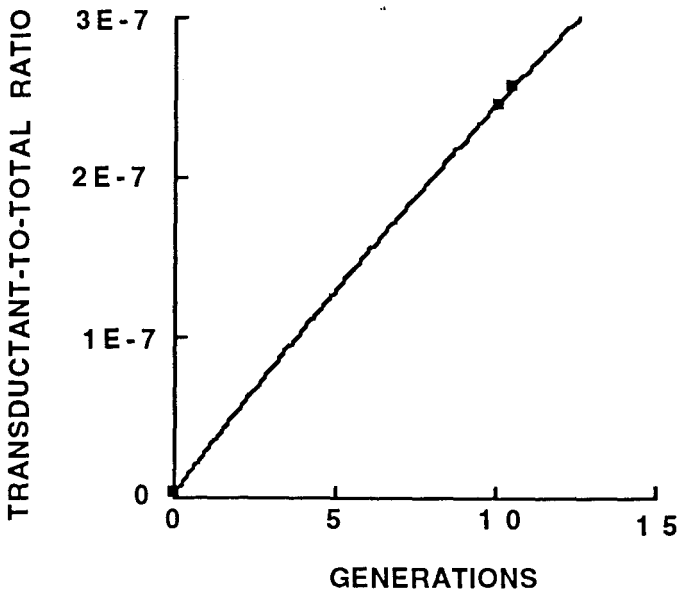
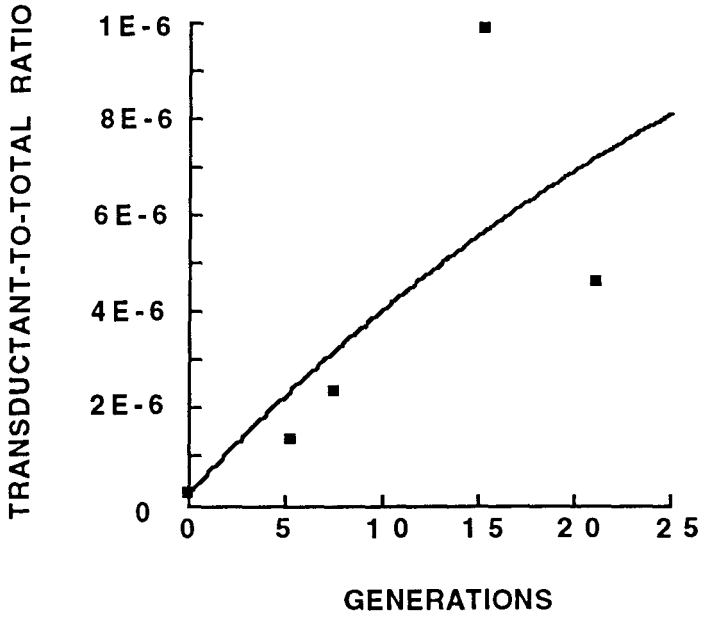


Fig. 36. Relative concentration of transductants observed in: (*a*) Experiment 8 and (*b*) Experiment 9. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

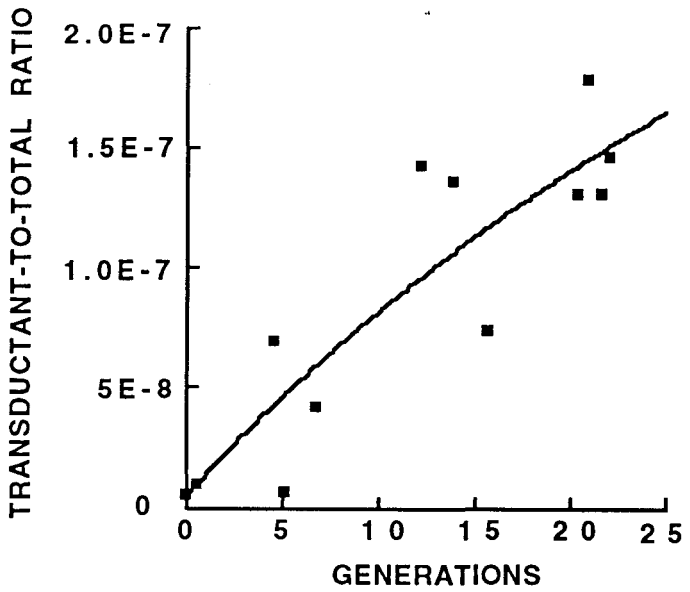


(*a*)

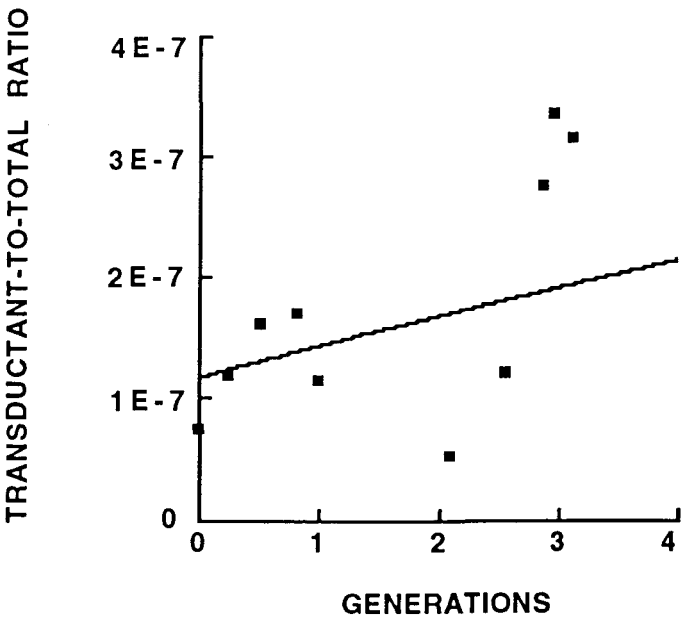


(*b*)

Fig. 37. Relative concentration of transductants observed in: (*a*) Experiment 10 and (*b*) Experiment 11. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

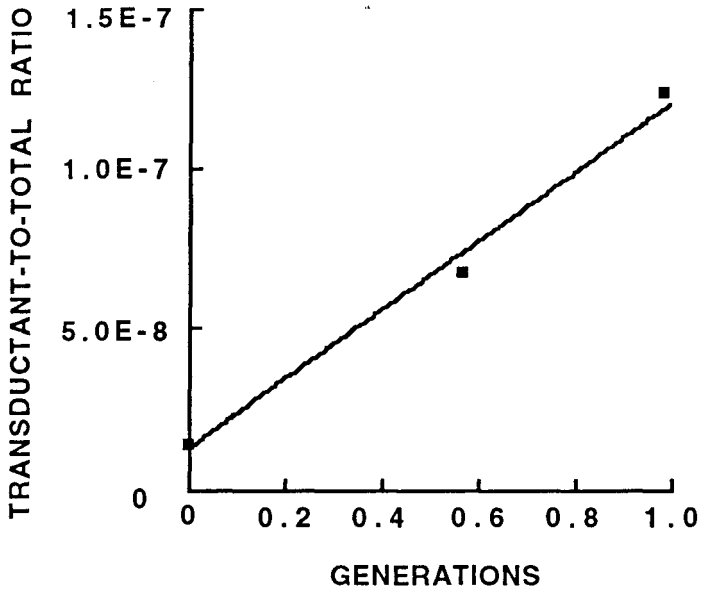


(*a*)

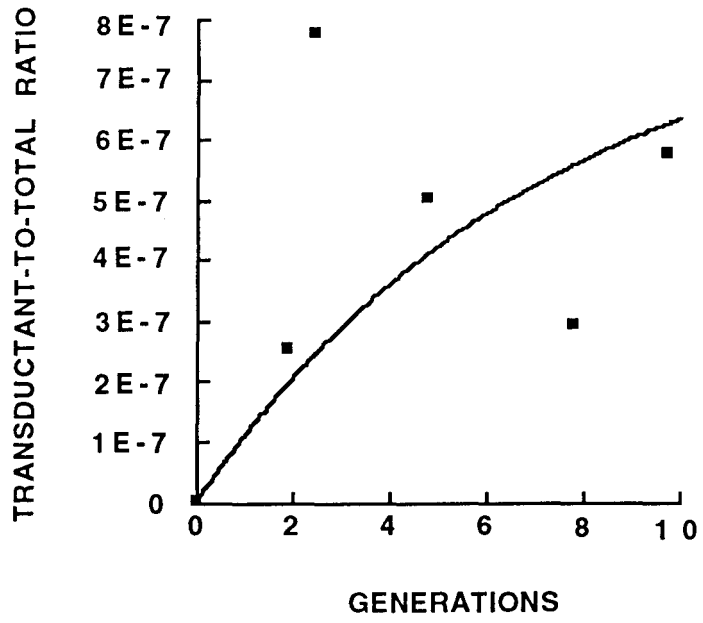


(*b*)

Fig. 38. Relative concentration of transductants observed in: (*a*) Experiment 12 and (*b*) Experiment 13. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.



(*a*)



(*b*)

Fig. 39. Relative concentration of transductants observed in: (*a*) Experiment 14 and (*b*) Experiment 15. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

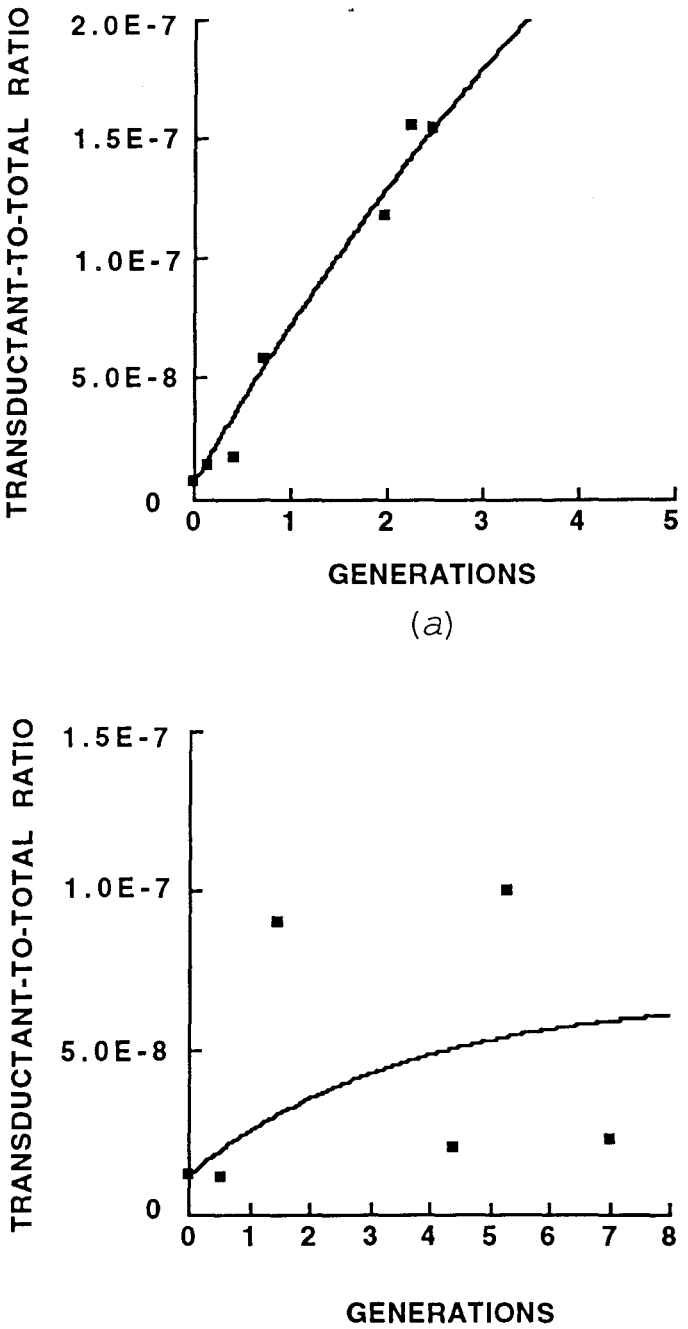


Fig. 40. Relative concentration of transductants observed in: (*a*) Experiment 16 and (*b*) Experiment 17. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

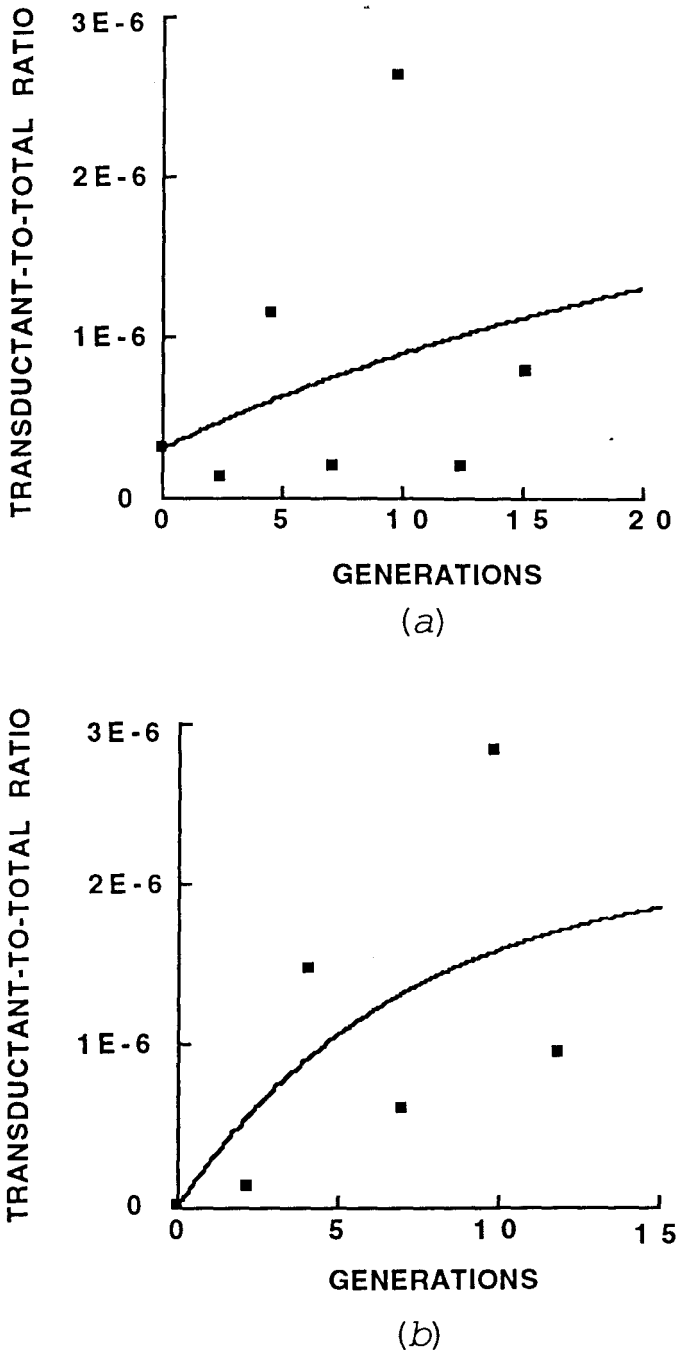


Fig. 41. Relative concentration of transductants observed in: (*a*) Experiment 18 and (*b*) Experiment 19. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

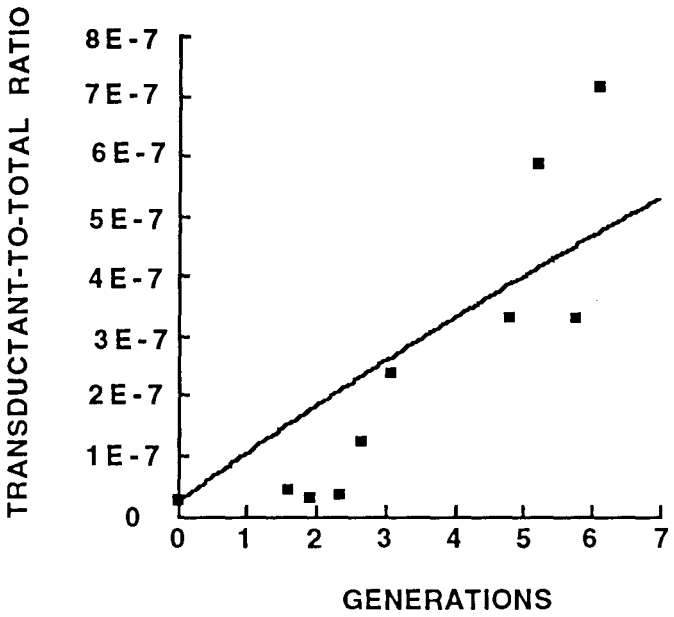


Fig. 42. Relative concentration of transductants observed in Experiment 20. Curves were generated by plotting Equation [25] using observed or calculated values for f, $(T/N)_o$, and $\Delta(T/N)_{trans}$.

In the previous chapter, equations were derived to describe how selection and transduction may affect the relative number of transductants in an emerging transductant population. Hypothetical curves calculated from the mathematical model suggested for circumstances of negative selection (f <1), the transductant-to-total ratio approaches a limiting value as given by,

$$\lim_{i \to \infty} (T/N) = \frac{\Delta(T/N)_{trans}}{1-f} \qquad [21]$$

where T is the concentration of transductants, N is the total cell number, $\Delta(T/N)_{trans}$ is the relative number of transductants formed per generation and f is the selection factor. As the transductant-to-total ratio approaches a limiting value, changes in the transductant-to-total ratio may be described by,

$$(T/N)_g = \frac{\Delta(T/N)_{trans}}{1-f} - \left[\frac{\Delta(T/N)_{trans}}{1-f} - (T/N)_o \right] e^{\ln(f)g} \qquad [25]$$

where g represents the number of generations.

Data obtained from growth rates in batch culture (Table 2) and control experiments (Table 5) suggest that the transductant strain experiences negative selection with respect to the total population. For each experiment, Equation [25] was solved for transduction rate, $\Delta(T/N)_{trans}$. Concentrations of transductants and total bacteria, and the

generations they were observed, were entered as variables into the equation. Values for the selection factor, *f*, were calculated from the selection coefficient, *s*, observed in a control experiment (Table 5) performed at a comparable generation time using the simple formula:

$$s = \ln f \qquad [19]$$

For each experiment, Equation [25] was solved for the best fit value of $\Delta(T/N)_{trans}$ using the nonlinear modeling function of SYSTAT. Table 8 gives computed values and standard errors for the relative transduction rate, $\Delta(T/N)_{trans}$. All values for $\Delta(T/N)_{trans}$ were positive, indicating that transduction was observed under all conditions tested. The values for transduction rate were within a range of 3.1×10^{-9} to 9.8×10^{-7} transductants formed per total population per generation per ml.

Also shown in Figures 36-42 are curves generated from Equation [25] using calculated values of $\Delta(T/N)_{trans}$. Comparison of the data points to the plotted curves provides a visual indication of the fit of Equation [25]. In some experiments, the curves describe changes in transductant-to-total ratios quite well (expts 10-12, 14, 16, 20). Likewise, in these experiments, calculations of the transduction rates, $\Delta(T/N)_{trans}$, had low standard errors (Table 8). In other experiments, curves did not fit the data points as closely

TABLE 8

Relative and apparent transduction rates calculated from mathematical model, Equation [25]

Expt	Sample size(n)	Relative Rate $\Delta(T/N)_{trans}$ ±s.e. $\frac{T}{N}$ ml gen	Equilibrium Total Conc N	Apparent Rate ΔT_{trans} $\frac{T}{}$ ml gen
High Cell Conc.				
8	8	$7.03 \pm 2.73 \times 10^{-9}$	7.30×10^{8}	5.13×10^{0}
9	3	$9.82 \pm 11.01 \times 10^{-7}$	7.37×10^{8}	7.24×10^{2}
Low Cell Conc. Low PBR[a]				
10	3	$2.69 \pm 0.01 \times 10^{-8}$	1.15×10^{6}	3.09×10^{-2}
11	5	$4.39 \pm 1.16 \times 10^{-8}$	1.78×10^{6}	7.81×10^{-2}
12	12	$8.85 \pm 0.73 \times 10^{-9}$	8.20×10^{5}	7.26×10^{-3}
13	10	$3.07 \pm 0.61 \times 10^{-9}$	4.06×10^{6}	1.25×10^{-2}
14	3	$1.09 \pm 0.05 \times 10^{-8}$	2.63×10^{6}	2.87×10^{-2}
15	6	$7.94 \pm 2.51 \times 10^{-9}$	3.24×10^{6}	2.57×10^{-2}
16	7	$6.69 \pm 0.28 \times 10^{-8}$	1.08×10^{7}	7.23×10^{-1}
17	6	$1.65 \pm 0.70 \times 10^{-8}$	1.81×10^{7}	2.99×10^{-1}
Low Cell Conc. High PBR				
18	7	$8.42 \pm 4.58 \times 10^{-8}$	1.17×10^{6}	9.85×10^{-2}
19	6	$2.77 \pm 0.76 \times 10^{-7}$	7.41×10^{6}	2.05×10^{0}
20	10	$8.31 \pm 1.27 \times 10^{-8}$	4.46×10^{5}	3.71×10^{-2}

[a] Phage-to-bacteria ratio

(expts 8-9, 13, 18). However, the processes of transductant formation and transductant washout are both random in nature. Also, differential killing of transductants by the cryptic phage may have occurred in experiments 8 and 9. Therefore, the reduced fit of the curves in some experiments may simply be due to random fluctuations in the transductant population as opposed to inappropriateness of the model.

The relative transduction rate, $\Delta(T/N)_{trans}$, is expressed as the rate of increase in transductants with respect to the total cell density. It would be useful to also examine the rate at which transductants themselves accumulate. This may be accomplished by multiplying the relative transduction rate, $\Delta(T/N)_{trans}$, by the total cell concentration at equilibrium, N. The resulting apparent transduction rate ΔT_{trans} is expressed in units of transductants formed per ml per generation. Values for apparent transduction rate are also listed in Table 8.

Factors that affect transduction

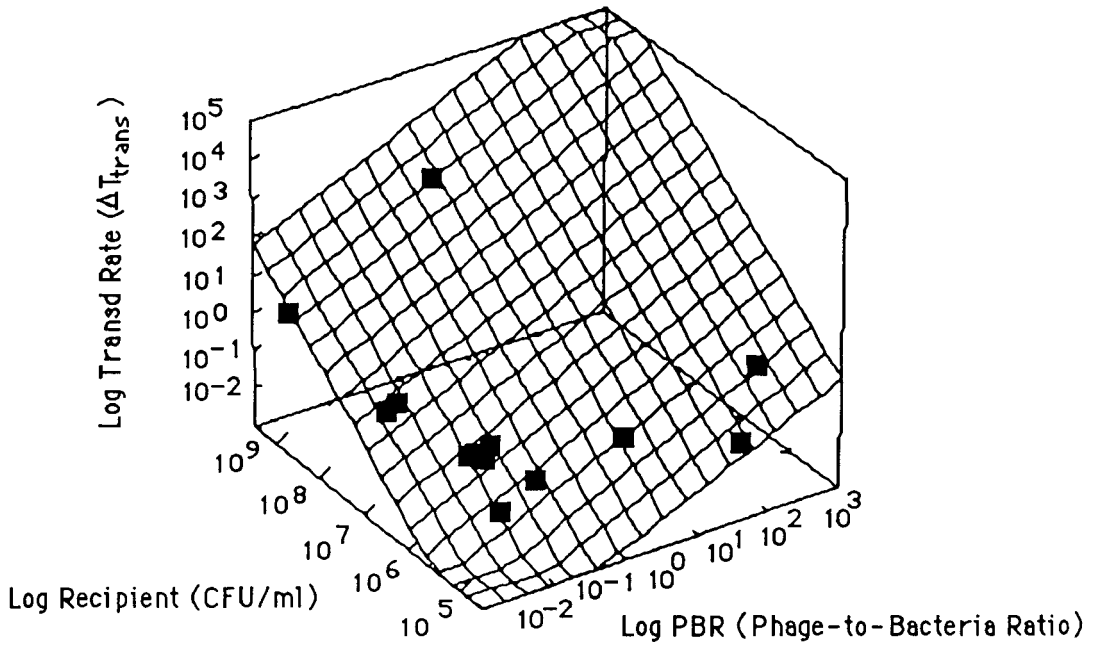
The transduction experiments were conducted over a range of conditions so that factors that affect transduction rate could be identified. Experimental variables examined to determine their potential influence on the apparent rate of transduction, ΔT_{trans}, include: donor, recipient, and phage concentrations, donor-to-recipient ratio, phage-to-bacteria ratio, and cell generation time. Variables were analyzed

after transformation into logarithms due to their large range in values. With SYSTAT, a multiple linear regression was performed using the apparent transduction rate as the dependent variable and the factors listed above as independent variables.

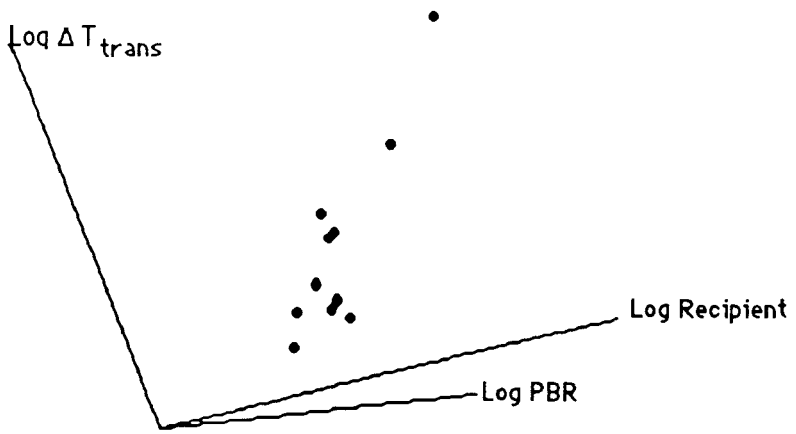
Only recipient concentration and phage-to-bacteria ratio showed any significant correlation to apparent transduction rate. Figure 43 illustrates this relationship, and gives the equation describing the contribution and standard error of each variable. The relationship was statistically significant, as indicated by the low *p*-value (< 0.05) and a high squared multiple correlation, R^2, (0.85).

The dependence of transduction rate on recipient concentration reflects an increased opportunity for transduction to occur when the recipients are abundant and thus more available for transduction. The correlation between transduction rate and phage-to-bacteria ratio (PBR) actually reflects a relationship with the transducing particle-to-bacteria ratio. The direct proportionality of these two ratios was indicated by batch studies, where a consistent proportion of total bacteriophage were transducing particles (Table 3). Therefore, a greater number of interactions between recipients and transducing phage can occur when the recipients are in abundance, and there are more bacteriophage (and thus transducing phage) per

ΔT_{trans}= 1.23 ±0.17 Log Total + 0.84 ±0.17 Log PBR $p = 9 \times 10^{-5}$ $R^2 = 0.846$



(a)



(b)

Fig. 43. Graphs illustrating the increase in apparent transduction rate with recipient concentration and phage-to-bacteria ratio: (*a*) an xy-cut plane best fit to the points representing experiments 8-20. (*b*) A two-dimensional representation of graph *a* rotated around the z-axis (transduction rate), and viewed from where the edge of the plane would lie.

bacterium. A greater rate of interaction would lead to a greater number of transduction events which occur.

In this system, the donor strain can intercept transducing particles. Transduction of the donor strain with plasmid-bearing transducing particles will not be detected because the donor strain already bears plasmid Rms149. Many of the transducing particles will be "lost" to donor bacteria and therefore not contribute to the measurable transduction rate. Therefore, transduction rate depends upon the ratio of transducing particles to total bacteria, not just recipients, because both donor and recipient strains are able to adsorb the transducing particles.

Experiments performed with the same concentrations of limiting nutrient (yeast extract), and variable dilution rates, yielded significantly different equilibrium concentrations of recipient cells (Table 6). Recipient density increased exponentially with cell generation time (Figure 44). This may be due to differences in nutritional requirements for cells growing at different rates. Greater amounts of substrate are required for rapid cell growth, whereas slow-growing cells need only enough substrate for maintenance metabolism (60). Therefore, increasing the generation time acted to increase the number of recipients available for transduction per generation.

When the donor-to-recipient ratio was increased, either in batch culture (Table 3) or continuous culture (Figure 45)

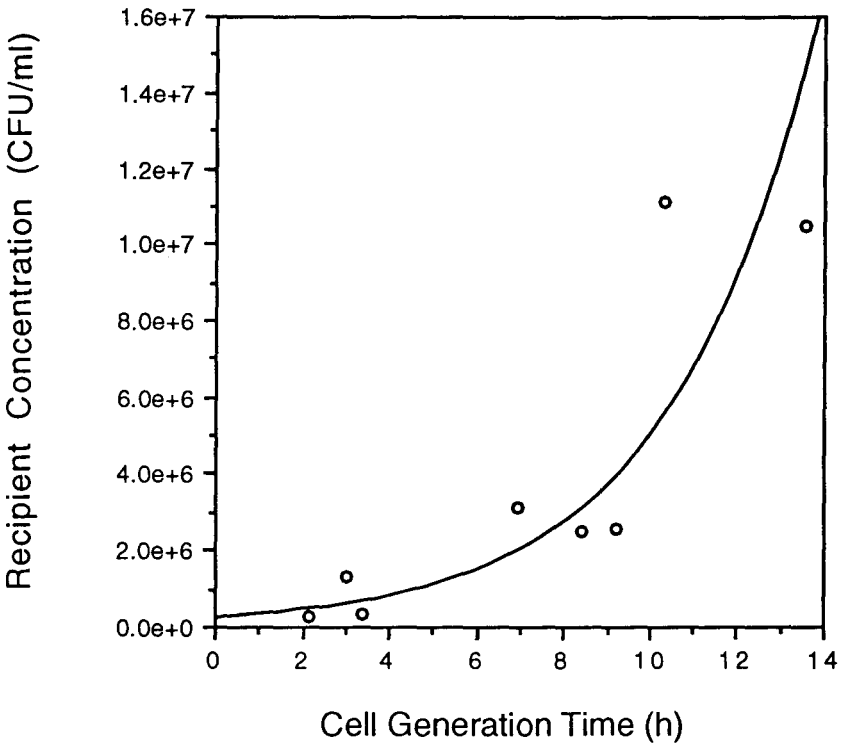


Fig. 44. Recipient concentration was observed to increase exponentially with increasing cell generation times. Experiments 10-17, performed using PMM-c supplemented with 4 x 10^{-5}% yeast extract.

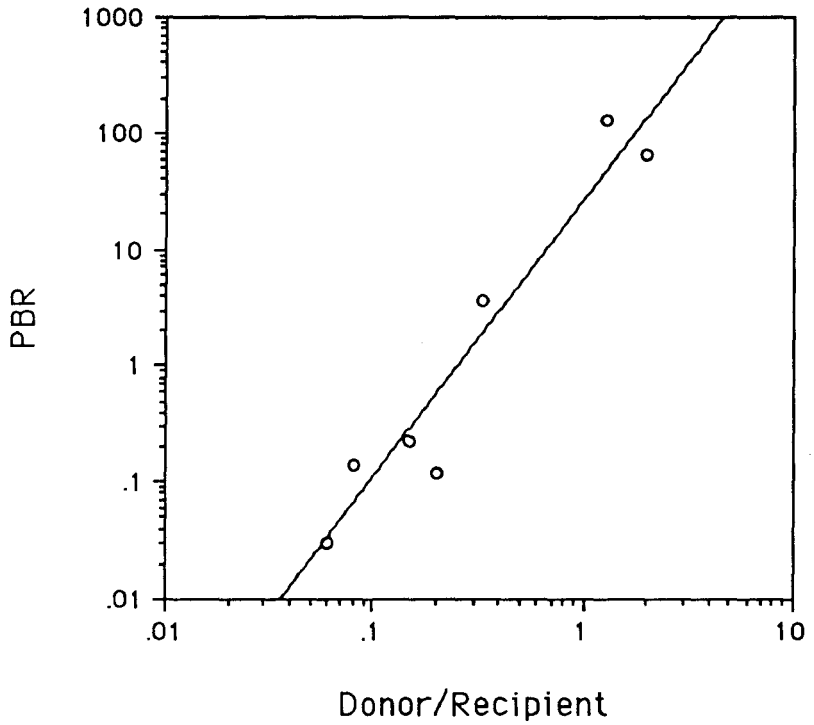


Fig. 45. Phage-to-bacteria ratio (PBR) was observed to increase with the donor-to-recipient ratio. Transduction experiments were performed at similar generation times and total cell concentrations (Expts 13-16, 18-20).

there was a significant increase in the phage-to-bacteria ratio (PBR) as well. This effect was due to a proportionately greater number of nonlysogens (*i.e.*, donors) available for lytic infection by bacteriophage F116. The relationship between nonlysogenic bacteria and phage concentration was previously noted by Ogunseitan *et al.* (49). Increasing the donor-to-recipient ratio in the chemostat experiments affected the frequency of transduction inasmuch as it altered the ratio of phage, and thus transducing particles, per bacterium.

CHAPTER VI

DISCUSSION

The results presented in this dissertation indicate that transduction can act to stabilize a novel phenotype in a bacterial population. This was determined by creating and testing a mathematical model which describes the contributions of transduction and selection on transductant populations. The model was tested in continuous culture experiments performed over a range of environmentally relevant conditions.

The first component of the mathematical model describes selection. Selection in competing continuous culture populations results in an exponential loss or gain in relative cell number with respect to time (7, 12, 34). Control experiments were performed to quantify the effects of selection alone on the transductants with respect to the total population. These experiments were conducted using nonlysogenic derivatives of the strains so that no new transductants could be formed.

Control experiments were conducted over cell generation times ranging from 2-13 h. In all cases, the mock-transductants were selected against. Values for the

selection coefficient, determined using Equation [17], indicated that selection per generation increased with generation time (Table 5). Dykhuizen (11) also observed this relationship in *E. coli* for generation times up to 3 h. Selection in continuous culture is often reported per hour, because generations overlap and the age of cells are equally distributed (7, 12). The rate of selection against the transductants per hour averaged -0.013, or 1.3% loss per hour. This is comparable to the rate of 2.7% loss per hour predicted from growth rate differences in batch culture.

Continuous culture experiments were conducted in a minimal salts medium supplemented with yeast extract as the sole nutritional source. Carbon concentrations in natural waters are typically 1 to 15 mg/L (65). In the chemostat experiments, yeast extract was provided over a range of concentrations, from 5×10^{0} to 9×10^{-8} mg yeast extract/L, with the majority of experiments conducted at 4×10^{-4} mg yeast extract/L. These conditions resulted in bacterial concentrations of 5×10^{5} - 7×10^{8} CFU/ml and bacteriophage densities of 3×10^{4} - 8×10^{8} PFU/ml. These concentrations are representative of natural levels of aquatic bacteria (4, 40) and phage (3, 14) found by direct count methods.

At high concentrations of yeast extract, a cryptic virus was induced from all strains examined, including the parental type strain PAO1. Terry and Mattingly (62) and Terry *et al.*

(63) also observed the induction of a virus from *P. aeruginosa* PAO1, which was able to plaque on the strain from which it originated. The cryptic virus was unable to transduce plasmid Rms149 to any significant degree, as indicated by control trials performed at high concentrations.

Chemostat populations were monitored for the establishment of lysogeny in the donor strain. Under all experimental conditions, some portion of the donors became F116 lysogens. At higher donor to recipient ratios most of the donors (>80%) became lysogens, probably as a result of higher phage concentrations. Although it seems that lysogeny would clearly be advantageous in a population containing phage, it was found that some percentage of the donors remained nonlysogenic. This may be due to the slight growth advantage found in nonlysogens over lysogens (Table 3).

Transduction experiments were performed over a range of concentrations, generation times and donor to recipient ratios. In an attempt to assess the contributions of selection and transduction, data from each experiment were entered as variables into the mathematical model:

$$(T/N)_g = \frac{\Delta(T/N)_{trans}}{1-f} - \left[\frac{\Delta(T/N)_{trans}}{1-f} - (T/N)_o \right] e^{\ln(f)g} \quad [25]$$

where T represents transductant concentration, N is total cell number, g is generations and f is the selection factor

determined from control experiments performed under similar conditions. Positive rates of transduction were calculated for all experimental conditions, indicating that transduction was causing higher levels of transductants in the populations (Table 8). Curves calculated from Equation [25] were compared to the relative number of transductants observed in each of the experiments (Fig. 36-42). The validity of the model is supported by the fit of many of the curves to observed changes in the transductant-to-total ratios. However, in some experiments, there was not a close fit of the calculated curves to the data points. This was attributed to the random fluctuations observed in these data, possibly caused by the random nature of transductant formation and washout.

The continuous culture experiments showed plasmid transfer rates of 10^{-6} to 10^{-9} per total per ml per generation between the plasmid-bearing donor and the lysogenic recipient. These frequencies are comparable to values of 10^{-6} to 10^{-8} transductants formed per recipient observed for F116-mediated transduction between *P. aeruginosa* strains in closed microcosm systems (47, 57). In initial experiments, where continuous flow began immediately, transductants appeared only sporadically at the limit of detection. The sporadic appearance of transductants was consistent with transduction rates observed in later experiments. The initial experiments

were conducted at 3 x 10^6 to 2 x 10^8 cells/ml for approximately 20 generations. These conditions would allow for the detection of 6 x 10^{-7} to 4 x 10^{-9} transductants formed per total per generation per ml. Because these values are similar to the relative rates of transduction, transductants were observed just at the limit of detection.

The apparent rate of transduction, or the rate at which transductants accumulate in the population, ranged from 10^{-2} to 10^3 transductants formed per ml per generation (Table 8). Apparent transduction rate increased significantly with both the recipient density and the phage-to-bacteria ratio (PBR) (Figure 43). The dependence on recipient concentration simply indicates an increased opportunity for transduction to occur when the bacterial strain which receives the transduced DNA is in greater abundance. The correlation between transduction rate and phage-to-bacteria ratio (PBR) probably reflects a relationship with the transducing particle-to-bacteria ratio. The direct proportionality of these two ratios is indicated by batch studies, where a consistent percentage of total bacteriophage were plasmid-bearing transducing particles (Table 3). Transduction proceeds more rapidly when there are greater numbers of phages, and therefore transducing particles, per bacterium. Furthermore, transduction rate would depend upon the ratio of transducing particles to total bacteria, not just recipients, because

both donor and recipient strains are able to adsorb transducing particles.

Even though transduction rate was found to be concentration dependent, a mass action model could not be directly applied to this experimental system due to the interfering presence of the donor strain. Plasmid-bearing transducing particles may adsorb to donor bacteria and therefore not contribute to the measurable transduction rate. Although the interaction of total bacteria and transducing phage may be governed by the law of mass action, it could not be measured in this system. Only transduction of the recipient strain was detectable. However, the validity of a mass action model could be tested by observing the rate of transduction when steady concentrations of transducing phage are added to recipient bacteria growing continuous culture.

Transduction rates were examined to determine the effect of altering the donor-to-recipient ratios. Increases in donor-to-recipient ratio caused corresponding increases in phage-to-bacteria ratio (PBR), reflecting a higher frequency of nonlysogenic bacteria available for the phage lytic cycle (Table 3, Figure 45). Therefore, in the experimental system, apparent rate of transduction was affected by donor-to-recipient ratio inasmuch as it influenced the PBR.

The concentration of recipients supported by growth medium was affected by the culture generation time. As

generation time increased, there was an exponential increase in equilibrium concentrations of recipients (Figure 44). This increase may be due to the lower nutritional needs of slow-growing bacteria, allowing for the subsistence of greater numbers of cells. Therefore, lengthening the generation time acted to increase the apparent transduction rate by increasing recipient cell density.

Information obtained from continuous culture experiments and the mathematical model suggest the importance of transduction as a mechanism of gene transfer and stabilization in the environment. In the chemostat conditions tested, transductants were at a selective disadvantage with respect to the total population. Under all experimental conditions, transduction served to stabilize the transductant phenotype by providing a continual source of new transductants to the population. The mathematical model predicts that for conditions of negative selection, the ratio at which transductants stabilize is affected by the degree of selection pressure (Figure 5). Computed transductant-to-total ratios approach a limit, as given by

$$\lim_{i \to \infty} (T/N) = \frac{\Delta(T/N)_{trans}}{1-f} \qquad [21]$$

Even under complete selection (f =0), transductants are predicted to stabilize at the level at which they are

created, the transduction rate $\Delta(T/N)_{trans}$. Therefore, transduction is important in that it can serve to counteract negative selection and act to stabilize an otherwise less fit phenotype.

Alternately, under circumstances of positive selection, the model predicts a continuous increase in the relative proportion of transductants (Figure 4). If all strains in a population had identical nutritional requirements, and the transductants maintained a competitive advantage, then the transductants could potentially outcompete the other bacterial strains present. The rate at which transductants increase with respect to the total population would depend upon the degree of the selective advantage (Figure 4).

In the mathematical model, the concentration of transductants in a population depends upon the rate of transductant formation. In situations of positive selection, greater transduction rates would cause the transductants to increase at more rapidly. If the transductants are under negative selection, greater transduction rates would increase the level at which transductants stabilize with respect to the rest of the population, as described by Equation [21] above (see Figure 6).

Transduction rate is affected by both the recipient concentration and the phage-to-bacteria ratio (Figure 43). Direct count methods indicate that bacteria are present at

concentrations of 10^6-10^7 cells/ml in aquatic environments (4, 40). Bacteriophage have been found at concentrations up to 10^8 PFU/ml in aquatic environments (3, 14). Additionally, many bacteria are known to harbor bacteriophage (20, 52). Observation of transduction in the chemostat under environmentally relevant conditions suggests its importance as a mechanism of gene transfer in nature. Because of its concentration-dependent nature, transduction would be especially prevalent in habitats where bacteria are present at very high densities, such as sewage, or waters polluted with excessive nutrients. Also, transduction would occur more frequently in microcosms where bacteria and bacteriophage congregate, such as on suspended particles (55) or on the surface of water bodies, at the liquid-gaseous interface (54).

The transduction experiments were performed at concentrations which can occur in the environment. Repeated attempts were made to obtain stable growth at even lower concentrations. However, *Pseudomonas* would either stabilize at approximately 10^6 cells/ml or wash out of the system. Dykhuizen experienced similar difficulties in stabilizing *E. coli* in the chemostat at low cell concentrations (personal communication). Kokjohn *et al.* (28) found that bacteria and bacteriophage can still undergo adsorption at and under concentrations found in nature. The present study suggests

that although the rate of transduction may be reduced at low cell densities, it should still occur, at a rate proportional to the recipient concentration and transducing phage-to-bacteria ratio.

The number of bacteria able to participate in transduction depends upon the types of transducing viruses present in the population and their host range specificity. Transducing viruses are often species-specific and DNA transduced between species may be susceptible to degradation by the recipient's restriction system (27). However, transducing phages have been found for numerous species of bacteria and are probably common in the bacterial world (27).

The frequency of transductional gene transfer from an introduced bacteria would be greatly increased if the organism released were the same species as a member of the indigenous population. If the species were represented in the indigenous population, then it is likely that there would be also be transducing viruses specific for that species in the population, either as free phages or associated with lysogens.

In this study, donor populations remained at a constant concentration, thus providing a steady source of transducing phage to the system. In an environmental release, the introduced strain may decrease over time due to competition with the indigenous population. Under these circumstances,

the engineered gene has the highest probability of being transduced when the strain is first introduced, and present at high concentrations. However, once an engineered gene is transferred to an indigenous organism, it may increase in frequency in this organism or be transferred further to other members of the population.

The long term stabilization of a gene in a population ultimately upon its selective value to an organism. The continuous culture experiments show that a novel genotype can stabilize in the population, even under negative selection pressure, provided that the source of the gene remains present and transducing particles are continually produced. If the donor organism were at a selective disadvantage, and not replenished, it would eventually disappear from the population. However, the gene may be transferred from the donor organism, before its disappearance, and stabilize in another bacterial strain. If the gene caused no selective disadvantage to the strain, it could be stably maintained.

When assessing the potential for transduction of genes from a genetically engineered bacteria, it is important to consider the circumstances of the release. Choosing a species represented in the indigenous population may not only increase its survival, but also its ability to participate in transduction. The chromosomal location of an engineered gene in the released organism does not prevent its transduction,

because unlike conjugation, transduction may transfer plasmid or chromosomal DNA. A large scale release would increase the persistence of not only the organism, but also its genes, by increasing the opportunity for transduction to occur (32). Concentrations of bacteria and phage at the chosen release site would also influence the rate of transfer, with greater rates of transduction occurring at sites having higher bacterial and phage densities. Increased fitness of the released organism would increase both its survival and its potential for transferring genes to the indigenous population. However, even if the organism chosen for environmental release is at a selective disadvantage, this does not necessarily reflect the fitness of its engineered gene(s). The genes may be transduced to an indigenous bacteria, where they may increase in frequency, or be transferred further to other members of the population.

APPENDIX

CALCULATION OF THEORETICAL CURVES

A computer program was written to calculate changes in the transductant-to-total ratio predicted by the equation

$$(T/N)_2 = (T/N)_1 f + \Delta(T/N)_{trans} \quad [20]$$

where $(T/N)_1$ and $(T/N)_2$ are the transductant-to-total ratios present at consecutive generations and $\Delta(T/N)_{trans}$ is the increase in the number of transductants per total per generation. This BASIC program is given in Figure 46.

```
10    INPUT "(T/N)o", T
20    INPUT "number of generations", N
30    INPUT "f and Δ(T/N)trans", F, D
40    PRINT "Generation", "T/N"
50    FOR I = 1 to N
60    T = T * F + D
70    PRINT I, T
80    NEXT I
90    END
```

Fig. 46. BASIC computer program for calculating changes in the transductant-to-total ratio for consecutive generations as predicted by Equation [21].

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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