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Cellstat—A continuous culture system of a bacteriophage for the study of the mutation rate and the selection process at the DNA level

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A bacteriophage is continuously cultured in the flow of the host bacterial cell under the control of a minicomputer. In the culture, the population of the noninfected cell is kept constant by the endogeneous regulation mechanism, so it is called the "cellstat" culture. Due to the high dilution rate of the host cell, the mutant cell cannot be selected in the cellstat. Therefore, the cellstat is suitable for the study of the mutation rate and the selection process of a bacteriophage under well-defined environmental conditions (including physiological condition of the host cell) without being interfered by host-cell mutations. Applications to coliphage fd, a secretion type phage, are shown as a measurement example. A chimera between fd and a plasmid pBR322 is cultured more than 100 h. The process of population changeovers by deletion mutants indicates that the deletion hot spots exist in this cloning vector and that this apparatus can be used also for testing instability of a recombinant DNA.

PACS numbers: 87.90. + y

INTRODUCTION

In 1950, Novick and Szilard[1] devised a chemostat, a continuous culture system of a bacterium for the study of the mutation rate. They observed the population changeovers by mutant *E. coli* and estimated the mutation rate. Continuous culture has more merits in quantitative studies than the serial transfer of batch culture because the environmental and physiological conditions are easily kept constant, and automation can be readily introduced. Although many data on mutation rate using chemostats have been accumulated, the results are not clear (e.g., the time/generation paradox[2]). One of the reasons for this situation may be that bacteria are too complex to study at DNA level. In 1967, Spiegelman *et al.*[3] performed an *in vitro* Darwinian evolution experiment using $Q\beta$ RNA and $Q\beta$ replicase. It was the first experimental study of molecular population dynamics in base sequence level.[4] As it was a serial transfer experiment, Eigen *et al.*[5] proposed an "Evolution Machine," a stirred flow reactor containing self-replicating RNA, that is, a kind, of continuous culture system. A simpler replicative molecular system, polyU-polyA replication by RNA-polymerase, was studied by Schneider *et al.* using a stirred flow reactor.[6] Although the *in vitro* system is a well-defined molecular system, its phenotype is not expressed by any translational products.

A small bacteriophage holds a position between bacteria and self-replicating RNA molecules in their levels of complexity. A bacteriophage has plenty of phenotypes. Recent remarkable development of DNA sequencing techniques, however, makes it possible to study the population dynamics of a small bacteriophage at the DNA base sequence level. A small bacteriophage can be utilized now as a well-defined molecular probe to the complex cell machinery.

In order to culture a bacteriophage continuously for the study of mutation rate and selection process, it is essential to regulate both the physiological and physicochemical conditions as precisely as possible since the mutation rate depends on the physiology of the cell.[2] In a chemostat culture, the physiological condition of the host cell fluctuates as discussed in the following section. A simple chemostat or a turbidostat in which both host bacteria and a phage are cultured, is not adequate because the host population is unstable due to coevolution by host–parasite interaction. A typical example is the case of emergence of a phage-resistant host cell.[7,8] This article reports the characteristics of an apparatus named "cellstat," which overcomes these difficulties, and some of its applications to a bacteriophage fd and its artificial recombinant.

I. DESIGN PRINCIPLES

A. Condition of the fresh host

A cellstat system consists of a turbidostat and cellstats which are connected to the turbidostat (Fig. 1). The turbidostat is a supplier of a physiologically constant host bacterial cell. For the turbidostat, a well-known relation[1]

$$k_g = D_T = F_T/V_T \tag{1}$$

holds between specific growth rate of the cell k_g, and the dilution rate $D_T = F_T/V_T$, where F_T is the flow rate and V_T is the volume of the culture. The cellstat is a culture vessel of a bacteriophage in a flow of the host cell. It is designed to satisfy the following inequality

$$k_g \ll D_c = F_c/V_c\,. \tag{2}$$

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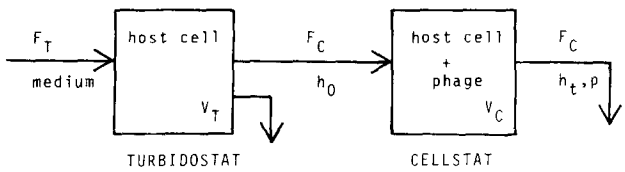


FIG. 1. Diagram of a cellstat system. V_T, V_c: Volume of the culture, F_T, F_c: Flow rate, h_0, h_t: Population density of the host bacterial cell, p: Population density of the bacteriophage. In the cellstat system, $F_T/V_T \ll F_c/V_c$ must be satisfied.

Under this condition, a mutant host cell which emerges in the cellstat is washed out rapidly. Therefore, selection of the mutant cell dose not occur in the cellstat. Inequality (2) is the condition of fresh host, which means that the mean residence time of the host in the cellstat is much shorter than its generation time, or that the physiological conditions of the host cell are kept constant by a large amount of influx of the host cell produced by the upstream turbidostat. Even under such conditions the bacteriophage is not washed out because the growth rate of the phage is much larger than that of the host cell.

B. External regulation

The turbidostat cannot be replaced by a chemostat in this system. In a chemostat culture, the physiological conditions of the host cell appear to be constant macroscopically; however, microscopically it must be fluctuating considerably because the endogeneous regulatory action in the chemostat originates from the buffering action of the large fluctuation between the log-phase state and the stationary-phase state.[9] Another characteristic peculiar to male-specific phage (e.g., bacteriophage fd) is that sex pili scarcely grow in the phase transition region used by a chemostat. Therefore, an external regulator is required to produce a steady host-cell flow, which is usually in the log phase.

The same discussion may be applied to the phage culture. The cellstat described here is endogeneously regulated by the host-parasite interaction. As discussed later, the cellstat becomes unstable as the population density of the phage decreases because its buffering action is weakened. At present we do not have any technique to monitor the phage population in real time. Therefore, a completely externally regulated system named "phagestat," which is ideal to serve our purpose, is beyond our capabilities.

A case of coliphage fd

As a quantitative treatment depends on the type of phage and operating conditions, here we limit our discussion to the case of bacteriophage fd on which actual experiments were performed. Extensive theoretical treatments appear elsewhere.[10]

Bacteriophage fd is a small filamentous single-stranded DNA phage of male *E. coli*. Its base sequence is known. These properties are suitable for the study of molecular population dynamics in the base sequence level. It is the secretion-type phage. This type is suitable for continuous culture under well-controlled environmental conditions, because the debris of lysed host cells is absent.[11]

The life-cycle of fd begins from the irreversible attachment to the tip of a sex pilus of the host.[12] There are many phenomenological kinetic models describing the host–parasite interaction and kinetic parameters reproducing the growth curve in batch culture. Here we chose a chemical reaction model among them, as shown in Fig. 2. P and H denote fd and noninfected host cell, respectively. The replicative form (RF) DNA is amplified exponentially in the cell in the X state. It secretes fd constantly in the Y state. The Z state is an imaginary state to monitor the condition of the fresh host. The population of Z should be negligibly small in the cellstat. The equations describing the population dynamics in the cellstat are as follows:

$$\begin{aligned}
\frac{dh}{dt} &= D_c h_0 - D_c h - k_1 hp + k_g h, \\
\frac{dx}{dt} &= \quad - D_c x + k_1 hp + k'_g x - k_2 x, \\
\frac{dy}{dt} &= \quad - D_c y \quad + k''_g y + k_2 x - k_4 y, \qquad (3) \\
\frac{dz}{dt} &= \quad - D_c z \quad + k'''_g z \quad + k_4 y, \\
\frac{dp}{dt} &= \quad - D_c p - k_1 hp + k_3 y .
\end{aligned}$$

Here h, x, y, z, and p are the population density of H, X, Y, Z, and P, respectively. All the coefficients have already been defined in Figs. 1 and 2. Total population density of the host cell defined in Fig. 1 is $h_t = h + x + y + z$. Equations (3) do not contain the variable corresponding to the growth-limiting factor of *E. coli*, and the specific growth rates have their maximum values, because *E. coli* must be cultured in the log phase in order to culture fd phage.

In the steady state, we obtain from Eqs. (3),

$$h = \frac{D_c}{k_1 k_2 k_3/(D_c + k_2 - k'_g)(D_c + k_4 - k''_g) - k_1}, \qquad (4)$$

$$p = h_0[k_2 k_3/(D_c + k_2 - k'_g)(D_c + k_4 - k''_g) - 1] - (D_c - k_g)/k_1 . \qquad (5)$$

Eq. (4) indicates that the population density of the noninfected cell is independent of the population density of

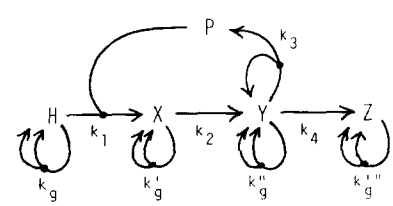


FIG. 2. A chemical reaction model of the host–parasite interaction for bacteriophage fd. P: fd, H: *E. coli*; X, Y, Z: three phases of the infected *E. coli*; k_g, k'_g, k''_g, k'''_g: specific growth rates; k_1, k_2, k_3, k_4: kinetic constants of each reaction step.

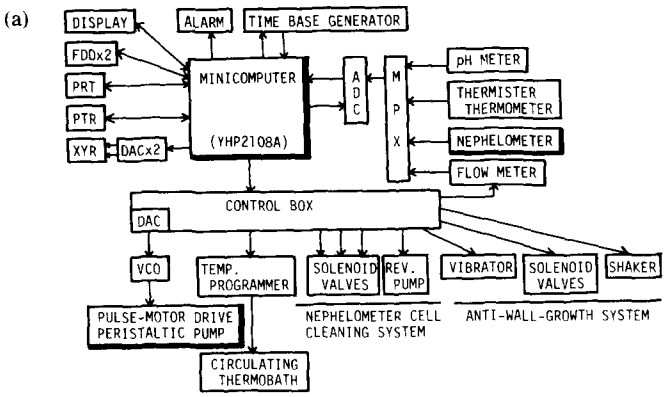
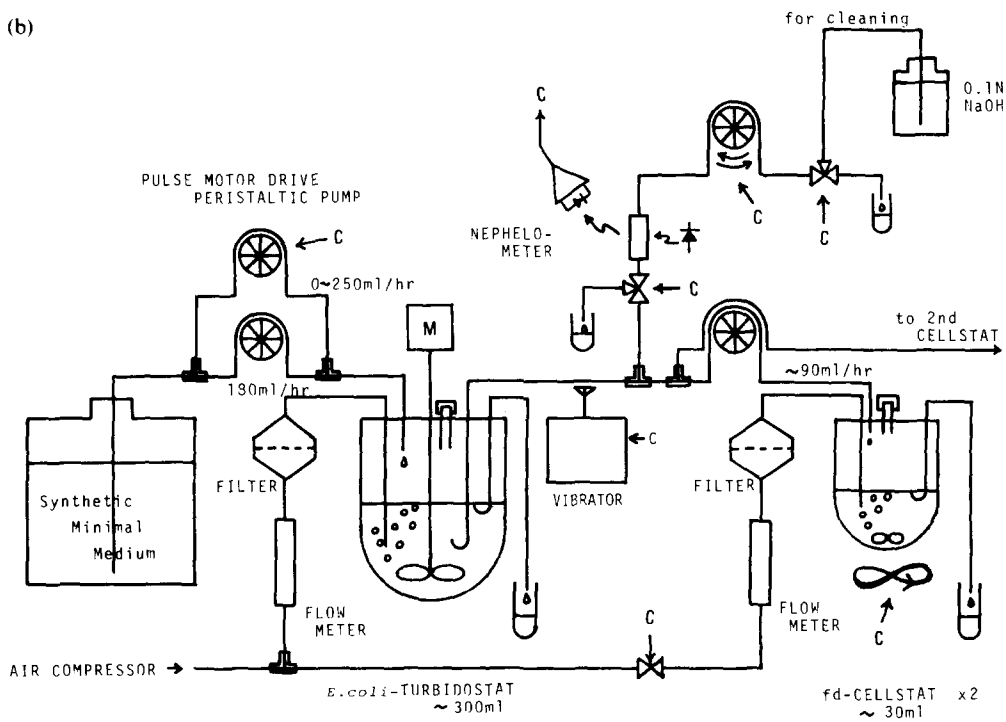


FIG. 3. Scheme of the cellstat system. (a) Computer system. MPX: Analog multiplexer (8 channel), ADC: Analog-to-digital converter (12 + 2 bit), DAC: Digital-to-analog converter (8 bit), VCO: Voltage-controlled oscillator. These devices are connected to a minicomputer (YHP2108*A*, 16 bit, 32 KW) via handmade interface cards. (b) Wet Devises. 'C' denotes the minicomputer. Wheel-like symbol denotes peristaltic pump. *M*: dc motor. ∞: 8-shape shaker.



the host cell in the influx flow. This fact corresponds to the situation that in the chemostat, the concentration of the growth-limiting factor is independent of the concentration in the influx medium flow.[1] Therefore, it is properly called "cellstat." The concept of cellstat is also applied to virulent phages.[10]

There is the maximum D_c value over which the steady-state solution [Eq. (5)] becomes negative, corresponding to the wash out of the phage. Thus we obtain the condition of existence:

$$D_c < D_{c,\max}(h_0)\,. \qquad (6)$$

Inequalities (2) and (6) are satisfied simultaneously when the host cell population density, h_0, is large enough, namely larger than 1×10^7/ml, which is calculated in the case of our standard experimental condition. This is a realistic value.

The stability of the steady-state solution Eqs. (4) and (5) is examined by the Lyapnov method and direct numerical analysis.[10] In the case of our standard experimental situation, the solution is asymptotically stable in the sense of Lyapnov. Direct numerical solutions of differential Eqs. (3) confirm the fact, although there is a negligibly small damped oscillation. (Examples are shown in Fig. 7.)

II. DESCRIPTION OF THE APPARATUS

A schematic diagram of the system is shown in Fig. 3. Culture vessels and tube system including the flow-nephelometer cell are sterilized in an autoclave (120 °C, 15 min). Air supplied by a diaphragm compressor (GAST, DOA-101) is sterilized through a HEPA filter and a millipore filter (pore size 0.22 μm). Contamination of fd phage through these filter systems has not been observed throughout three years of experimentation. A minicomputer (YHP2108A, 16 bit, 32 KW) calculates the optimum medium flow rate of the turbidostat (F_T) based on the data from the in-line flow nephelometer and outputs the data to drive a pulse-motor-drive peristaltic pump (handmade, motor: Nippon pulse motor PA61, tube: Scientific Industries Co. special silicon rubber tube). The principle of the control program is propor-

tional control in which the set value is shifted by an integrational control to counterplot against the wall growth effect.

The vessel of the turbidostat consists of a separable five-necked flask (500 ml) with a water jacket. Stirring is performed by a magnetically coupled vacuum stirrer. A U tube, the only exit to atmospheric pressure, keeps the water level and serves as a sampling port. Through another U tube made of Teflon, the host cell in log phase is transferred to two cellstats by a high-performance peristaltic pump (1.5% fluctuation, Harvard Apparatus 1201 using Tygon tube). Transfer tubes are made of Teflon (i.d. 1 mm) except in the pump. Cellstats consist of a separable round-bottom flask (50 ml) with a water jacket and a lid made of stainless steel (SUS 316) with various ports. Stirring is performed intermittently by an eight-shape shaker (Tokyo Rika, SS80) and constantly by a magnetic stirrer on it. The water level is kept constant by a tube which serves as a sampling port and is the only exit to the atmosphere. This level sensor is blocked when the shaker is working intermittently.

A. Anti-wall growth system

Wall growth and floc formation are the major technical problems to be solved in continuous culture. Teflon tubes used to transfer the host cell are vibrated by a solenoid (about 30 Hz). The above mentioned shaker serves as an anti-wall growth system for cellstats. Small glass tips sweeping the round bottom are also effective against the wall growth on the cellstat. Besides these physical methods, there is a biological anti-wall growth method using a pililess strain discussed in Sec. IV.

B. Time sharing flow nephelometer with cell cleaning system[13]

The light source of the nephelometer is an LED (635 nm, Monsanto, MV5152) flashing at 610 Hz. The scattered-light detector is a silicon photo diode (Hamamatsu TV, S789-5BK) which forms an angle of 27° with the incident light. An I-to-V converter (using CA3140) is located just behind the detector. A lock-in amplifier of our own design processes the signal and outputs it to the minicomputer via an A-to-D converter (Datel, HZ12BGC, effectively 14 bit operation by computer-gain control). This flow nephelometer can detect the population density of host cell from 3×10^5 to 3×10^9/ml.

In order to avoid wall growth and depositing of the floc, an alternating cleaning–sampling mechanism is introduced. 0.1 N NaOH solution flows every 5 min through the flow cell for 2.5 min to clean it and to give the offset intensity of scattered light. The computer memorizes this offset value and subtracts from the value of the scattered light intensity of the sample flow (Time Sharing System).

C. Temperature control and detection

Temperature is controlled by circulating the water in the jacket from a circulation thermobath (HAAKE, F2S). By a computer on-line Wheatstone bridge,[13] the temperature of the culture can be changed at a rate of up to 1 °C/min. Temperature is detected by the thermister inserted into a detection sleeve after autoclaving the vessel.

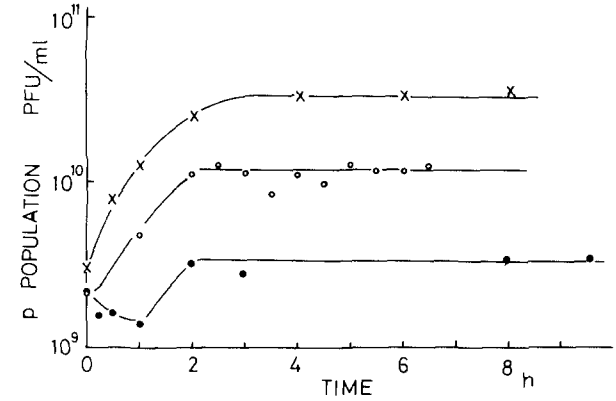


FIG. 4. Time course of the cellstat culture of bacteriophage fd wild type at various population density of the input host *E. coli* S26 (h_0). PFU: plaque forming unit; X: $h_0 = 1.9 \times 10^8$/ml, ($D_c = 2.2$ h^{-1}); O: $h_0 = 1.0 \times 10^8$/ml, ($D_c = 2.3$ h^{-1}), ●: $h_0 = 5 \times 10^7$/ml, ($D_c = 2.34$ h^{-1}) 37 °C, Davis minimal medium with vigorous aeration.

III. MATERIALS AND METHODS

Bacteriophage: Bacteriophage fd was a gift from Prof. Y. Okada. Single plaque isolation was performed for our stock strain. Bacteriophage fd107, an artificial chimera between fd and a plasmid pBR322, was a gift from Prof. H. Schaller.[14] Stock strain was produced by short time batch culture (*E. coli* S26 as a host) in Davis medium and PEG purification.[15]

E. coli: *E. coli* S26 (K38) was a gift from Prof. Y. Okada. Several single colony isolations were performed for our stock strains. *E. coli* K12 JE3100 was a gift from Dr. M. Tomoeda.

Enzymes and other materials: Restriction enzyme Hae III was purchased from Takara Shuzo, Co. Davis minimal medium was made with doubly distilled water and special grade reagents.

Cellstat culture: Standard operating conditions were as follows: Host: *E. coli* S26; Medium: Davis minimal medium; Temperature: 37 °C, $h_0 = 1$–2×10^8/ml, $V_T \sim 300$ ml, $D_T \doteqdot 0.8$ h^{-1}, $V_c \sim 30$ ml, D_c was around 3.0 h^{-1}, initial multiplicity of infection ~10, with rigorous aeration and stirring.

Population density measurement: For the phage, the standard double agar-layer plating method was employed using *E. coli* S26 as an indicator. As auxiliary method, HPLC (Toyo soda, HLC803 with a gel permeation chromatographic column, G-4000SW) was used, monitoring the absorbance at 270 nm. HPLC was used also to monitor constancy of the chemical environment using the G-4000SW and an anion exchange chromatographic column (IEX-520 QAE). For the host *E. coli*, turbidity at 660 nm using a spectrophotometer (HITACHI, type 200) was used after the calibration by microscope counting of the number of the cell.

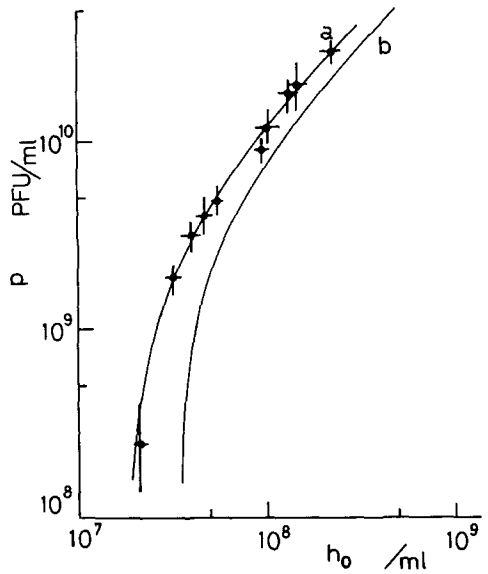


FIG. 5. Relation between the steady-state population density of fd (p) and the input population density of *E. coli* (h_0). The solid line is the theoretical curve with D_c = 2.4 h^{-1}(a) and D_c = 3.0 h^{-1}(b). The other parameters: $k_1 = 6 \times 10^{-10}$ (population/ml)$^{-1}h^{-1}$, k_2 = 1.8 h^{-1}, k_3 = 690 h^{-1}, k_4 = 0.48 h^{-1}, k_g = 0.96 h^{-1}, k'_g = 0.3 h^{-1}, k''_g = 0.6 h^{-1}, k'''_g = 0.91 h^{-1}. Experimental data (●) with various D_c's are shifted in order to reduce to the case of D_c = 2.4 h^{-1}.

Electron microscopy and length measurement: Purified phage by PEG method was stained negatively with phosphotungstate and observed by an electron microscope (HITACHI, H700H). Relative length of phages is measured on the digitizer tablet (YHP 9874).

Gel Electrophoresis: Single-stranded (ss) DNA of fd, its recombinant and its mutant, were purified by standard phenol extraction method. The ssDNA was digested by restriction enzyme *Hae III* (72 unit/μg DNA, incubated in standard reaction buffer at 37 °C for 20 h), and electrophoresed following the method of Peacock and Dingman[16] with a slight modification. 1-mm-thick slab gels were used and the concentration of the tray buffer was reduced to one third of that reported.

IV. RESULTS AND DISCUSSIONS

A. Performance of the turbidostat

General performance of the turbidostat is reported elsewhere.[17] The constancy of the cell population depends on the ability of wall growth and/or floc formation. *E. coli* K12 JE3100 having no *I* pili scarcely grow on the wall and scarcely form any floc. The standard deviation of the turbidity was 0.3% of the set value throughout 50 h. *E. coli* S26 in the transition phase is in the same situation. However, for *E. coli* S26 in the log phase which has *I* and *F* pili and which is apt to adhere to the wall, it requires very careful operation to keep the standard deviation of the turbidity within 1% of the set value.

An experiment on the temperature dependence of the specific growth rate of *E. coli* was carried out employing this system. It was shown that *E. coli* population immediately follows the change of temperature (up to 1 °C/min) between 23 ° ~ 38 °C. This means that the after effect of the cooling of *E. coli* during transfer from the turbidostat to cellstats is negligible. Data from the HPLC showed that the chemical environment was constant.

B. Performance of the cellstat

The wall growth of *E. coli* in the cellstat was reduced to 5% of total population.

For wild-type fd, the steady state is reached within 3 h after infection (*m.o.i.* = 10) (Fig. 4). The steady-state population (p) is plotted against the host cell population in influx (h_0) in Fig. 5. These data indicate that the cellstat is operated as was designed. The standard deviation of the steady-state population is within 20% of the mean value for wild-type fd throughout 40 h. Considerably large fluctuation may be due to the floc in influx, which is formed in the Tygon tube of the peristaltic pump, where the effect of the vibrator is weakened.

C. Population changeovers by the deletion mutants of a fd recombinant (fd107)[18]

The steady-state population (p) in the cellstat is given by Eq. (5). The mutant phage may have different k_i values. The indirect selection coefficient S^α of α-th mutant is defined by[19]

$$S_i^\alpha = (k_i^\alpha - k_i^0)/k_i^0 , \qquad (7)$$

where k_i^α, k_i^0 is the i-th kinetic constant of mutant and wild type, respectively. For simplicity, we postulate $S_i^\alpha = S^\alpha$ for i = 1, 2, 3, $S_4^\alpha = 0$. The relation between S^α and p^α is shown in Fig. 6.

Therefore, when the population changeover from α-th to β-th mutant occurs in the cellstat ($S^\alpha < S^\beta$), the total population increases from p^α to p^β.

A typical example was demonstrated when a chimera phage fd107 was cultured. Filamentous phage fd107 has a recombinant DNA (10770 base) between fd DNA (6408 base) and a plasmid pBR322 (4362 base). The

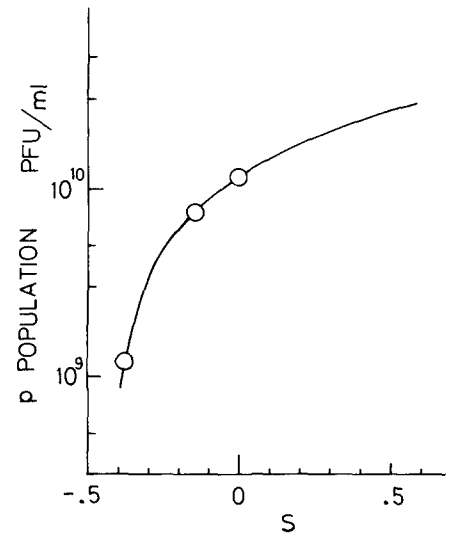


FIG. 6. Relation between the steady-state population density of fd (p) and the indirect selection coefficient (S) defined by Eq. (7). Circles represent fitted points of the experimental data in Fig. 7. (D_c = 3.0 h^{-1}, $h_0 = 1.8 \times 10^8$/ml).

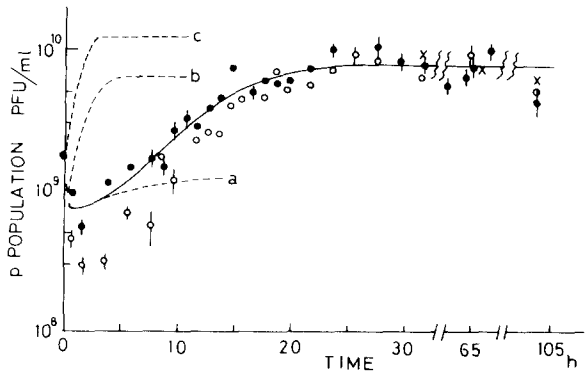


FIG. 7. Time course of the cellstat culture of an elongated fd recombinant (fd107) demonstrates population changeovers by the deletion mutants. Two independent cultures (○: CI and ●: CII) have a common host source ($h_0 = 1.8 \times 10^8$/ml). $D_{CI} = 3.1\ h^{-1}$, $D_{CII} = 3.0\ h^{-1}$. ×: Population density of the phage in CI obtained the data from HPLC. Broken lines are theoretical curves of the homogeneous nonmutable population having various indirect selection coefficients (S). (a): −0.38, (b): −0.18, (c): 0.0, i.e., wild type. The solid line is a theoretical curve for CII (●) based on the highly-mutable four-membered population *P*1(=fd107) ($S = -0.38$), *P*2($S = -0.30$), *P*3($S = -0.21$), *P*4(=fd107-111) ($S = -0.13$) with mutation rates $w_{21} = 2.6 \times 10^{-4}$, $w_{32} = 2.3 \times 10^{-5}$, and $w_{43} = 1.3 \times 10^{-5}$/replication.

region from pBR322 is not essential in order to be a bacteriophage. Therefore, the length of the elongated phage may decrease gradually by the population changeovers by deletion mutants, just as $Q\beta$ RNA in Spiegelman's experiment.[3] In Fig. 7, two time courses of the cellstat cultures CI and CII, into which the same host cell suspension flowed, is shown. The difference is caused by the different dilution rates of the two cellstats. The final steady state was not reached until 25 h after infection. If the population had been homogeneous and nonmutable, the steady state would have been reached more rapidly as indicated in the broken lines, and quite different curves would have been obtained when the initial contamination was small enough as in this case. These curves should be explained as a behavior of the highly mutable population. The fact that two independent cellstats gave similar curves means that the phenomena may be deterministic, that is, advantageous mutations occurred very frequently.

The following two indications show that these mutations are the site-specific deletion mutations. First, the length distribution of the filamentous phage determined by electron microscopy shows that the ratio of length of the fd wild type, fd107, and the finally dominant phage (fd107-111) is 1 : 1.7 : 1.22. 1.7 corresponds to the degree of polymerization of the elongated DNA.

Second, restriction fragments by *Hae III* of ssDNAs of the finally dominant phage in independent experiments show the same patterns in polyacrylamide gel electrophoresis. Comparing the pattern of fd107-111 with that of fd107, one can see that many bands are lost, but three new bands emerge. The fact is compatible with the postulate that the successive three deletions occurred. The fact that the finally dominant phages were not the wild-type fd is explained by absence of the specific deletion site (deletion hotspot) at the insertion site of pBR322.

The solid line in Fig. 7 is a theoretical curve for CII based on this postulate. Newly required microscopic kinetic parameters, that is, the indirect selection coefficients S^{α}, and the deletion mutation rate $w_{\beta\alpha}$ is determined by curve fitting. Obtained are:

$$S^{fd107} = -0.38, \quad S^{fd107\text{-}111} = -0.13$$

$$w_{\beta\alpha} = 10^{-4} \sim 10^{-5}/\text{replication}.$$

An artificial recombinant may not be stable from the stand point of the equilibrium between the mutation and the natural selection. This experiment demonstrates such instability. The time course of the increase of the total population can be regarded as a biological relaxation profile from the artificial nonequilibrium state to a selection equilibrium. Although many genetic engineers pointed out such instability, a quantitative treatment of the relaxation process under well-defined environmental condition has become feasible by the cellstat.

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