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# Quantitative Determination of Bacterial Replication In Vivo

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**A new methodology which permits the quantitative measurement of absolute bacterial replication in vivo is proposed. Mice were inoculated with mixtures of temperature-sensitive mutants and parental wild types, and the changes in the ratios of the two strains were measured. The number of wild-type generations was calculated from the declining ratios over time with the formula $n = \log (r_0/r_t)/\log 2$; $n$ is the number of generations, and $r_0$ and $r_t$ are the ratio of temperature-sensitive mutants to the parental wild type at time zero and at the times sampled throughout the experiment. The replication rate was determined by regression analysis. A mathematical argument for the formula is presented. Using this technique, we determined the mean generation times of *Escherichia coli* (33 min) and *Pseudomonas aeruginosa* (20 min) in the peritoneal cavities of mice, in the face of host clearance mechanisms during the first stages of infection.**

The major difficulty encountered in studies on the in vivo replication of microbial pathogens is the concomitant clearance of organisms by the many defense mechanisms of the host. Several investigators in the past have proposed models for the quantitative measurement of replication rates in animals with some success, although these models are limited to rather specialized bacterial systems (3, 5–7). A relatively simple solution to the problems of studying in vivo replication is the inoculation of temperature-sensitive (ts) mutants in combination with the wild-type (wt) parents and the evaluation of changes in the ratio of the two organisms. We demonstrate here the feasibility of this quantitative model with ts mutants of *Escherichia coli* and *Pseudomonas aeruginosa* during the first stages of infection with their respective wt. In contrast to most previous methods for measuring in vivo replication, this model is potentially applicable to almost any bacterial pathogen and avoids the problems associated with radiolabeled bacteria.

## MATERIALS AND METHODS

**Bacterial strains.** The mutants were isolated after chemical mutagenesis of *E. coli* (strain Easter) and *P. aeruginosa* (Fisher-Devlin-Gnabasik immunotype 1) by protocols described elsewhere (8, 9). The mutants E/2/64 (*E. coli*) and D/1/8 (*P. aeruginosa*) cease replication immediately after transfer to the nonpermissive temperature (≥36°C) and remain completely viable under these conditions for at least 18 h. The parental wild types, on the other hand, replicate well at 36 to 40°C (8, 9). A multiple-antibiotic-resistant strain of *P. aeruginosa*, MAR, was derived by sequential plating on media containing streptomycin, nalidixic acid, erythromycin, and novobiocin. A revertant of D/1/8 was obtained by plating $>10^9$ CFU and incubating at 36°C. The reversion rate for D/1/8 to $ts^+$ is $10^{-8}$.

**In vitro experiments.** Experiments were first performed in vitro with mixed cultures of the ts mutants and wt to establish that neither strain interfered with the viability or growth profile of the other. Mixtures of ts mutants and wt were inoculated at ratios of 5:1 into broth cultures at 29°C and shifted in mid-log phase to 40°C. At various times thereafter, samples were taken from the cultures, diluted appropriately, plated, and incubated at 29°C (for total viable cells) and 37°C (for wt CFU). The numbers of viable ts mutants were obtained by subtraction, and ratios of ts mutant to wt were calculated and plotted as a function of time. The number of generations, $n$, of wt was plotted as a function of time, and the mean generation time (MGT) was estimated on the basis of the formula $n = \log (r_0/r_t)/\log 2$; $r_0$ and $r_t$ are the ratio of ts mutant to wt at times zero and $t$. The derivations of this formula and of the MGT are given below.

**In vivo experiments.** Replication of the pathogens in vivo was studied with mixtures of mutants and wt parents inoculated intraperitoneally into groups of outbred, 3- to 5-week-old ICR mice. Immediately and at various times thereafter, groups of three to five mice were sacrificed by cervical dislocation, and the peritoneal cavities were lavaged with 5 ml of saline. The volumes recovered were immediately diluted appropriately in sterile distilled water, plated, and incubated at two different temperatures to determine the numbers of viable ts mutants and wt present in the samples. The ratios of mutant to wt CFU were then plotted as a function of time, and the number of generations of the wt and the MGT were calculated from the formula given above.

Experiments were also performed with the lungs of mice exposed to aerosols of mixtures of ts and wt *P. aeruginosa* or ts mutant and a revertant. The procedure is described in detail elsewhere (11). Briefly, mouse lungs received doses of $1 \times 10^4$ to $2 \times 10^4$ total CFU. At time zero and various times thereafter, the animals were sacrificed, and the lungs were removed aseptically and homogenized in 5 ml of cold, sterile distilled water. Samples were diluted appropriately, plated, and incubated at the permissive and nonpermissive temperatures for the determination of viable ts and wt numbers.

**Mathematical considerations.** The formula for the number of generations of wt, applicable both in vivo and in vitro at nonpermissive temperatures, is derived as follows.

Let $m$ and $w$ denote the number of viable ts mutants and wild-type organisms, respectively, let $G_m$ and $G_w$ be their growth rates, and let $D_m$ and $D_w$ be their death rates. Our definition of death here includes all mechanisms which reduce the viable population, i.e., physical removal to the blood or other organs and death in situ. Then $dm/dt = (G_m - D_m)m$, and $dw/dt = (G_w - D_w)w$. At the nonpermissive

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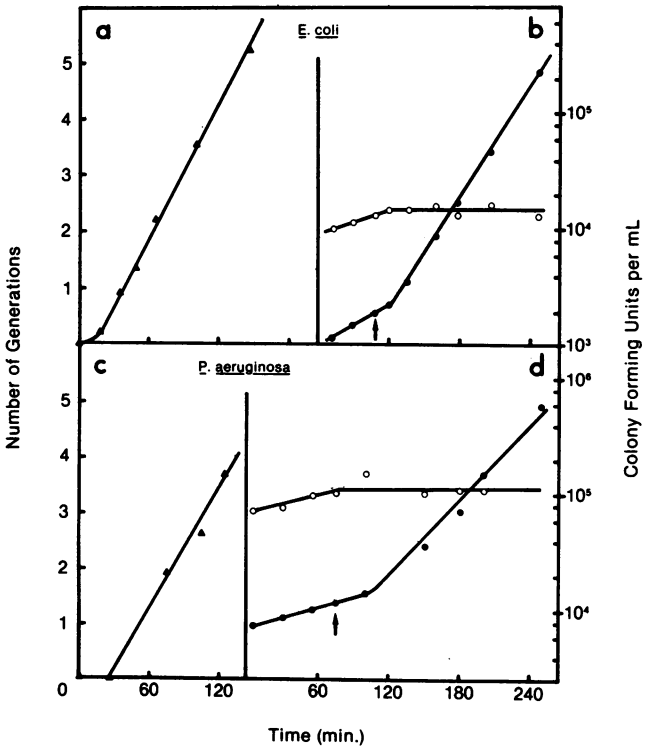


FIG. 1. Growth curves of *E. coli* and *P. aeruginosa* in mixed culture with their respective ts mutants in vitro. (a and c) The number of generations of *E. coli* and *P. aeruginosa* versus time in mixed culture with their respective mutants in vitro. The data points were determined from the ts/wt ratios in various samples. Time zero in these panels corresponds to the time of temperature shift, indicated by the arrows in (b) and (d). The linear portion of the curve was then determined by regression analysis [$r = 0.970$ and 0.993 in (a) and (c), respectively]. The reciprocals of the slopes give MGTs of 26 and 28 min in (a) and (c), respectively. (b and d) CFU per milliliter of ts mutants (○) and wt (●). At the time indicated by the arrow, the cultures were shifted from 29 to 40°C.

temperature, $G_m = 0$. If the ts mutant and the wt are cleared at the same rate, then we can take $D = D_m = D_w$ to be their common death rate (in vitro, $D \simeq 0$ for the first few hours). Then

$$\frac{dm}{dt} = -Dm \tag{1}$$

$$\frac{dw}{dt} = (G_w - D)w \tag{2}$$

Regarding $D$ and $G_w$ as possibly unknown functions of time, we integrate (1) and (2) to get

$$m_t = m_0\, e^{[-\int_0^t D(s)\,ds]}$$

and $$w_t = w_0\, e^{[\int_0^t G_w(s)\,ds - \int_0^t D(s)\,ds]}$$

Calculation of the ratio $r = m/w$ at times zero and $t$ yields

$$\frac{r_0}{r_t} = e^{[\int_0^t G_w(s)\,ds]} = 2^n \tag{3}$$

The middle expression in (3) is the number of wt bacteria at time $t$ derived from a single bacterium at time zero in the absence of clearance or death. This quantity is also equal to $2^n$; $n$ is the number of generations at time $t$. Thus,

$$n = \frac{1}{\log 2}\int_0^t G_w(s)\,ds = \frac{\log (r_0/r_t)}{\log 2} \tag{4}$$

This formula is independent of the clearance mechanisms, provided that they act equally on both the ts mutant and the wt.

To calculate the MGT, note that initially the wt may need to adjust to its new environment. The growth rate $G_w$ climbs to a steady value $G$ after a time $L$, the "lag" time. So

$$G_w(t) \equiv G, \text{ for } t \geq L \tag{5}$$

The MGT is the time $T$ required for the wt population to double thereafter. From (3) and (5) we obtain

$$2 = \frac{2^{n+1}}{2^n} = \frac{e^{[\int_0^{t+T} G_w(s)\,ds]}}{e^{[\int_0^t G_w(s)\,ds]}} = e^{[\int_t^{t+T} G_w(s)\,ds]} = e^{GT}$$

Hence, $G = (\log 2)/T$. Substituting this result into (5) and (4) and integrating, we find that for a suitable constant $k$ and for $t \geq L$

$$n = k + t/T \tag{6}$$

so that the graph of $n$ versus $t$ has a linear portion. The MGT is the reciprocal of the slope of this linear portion, and the slope can be estimated graphically or by regression analysis.

## RESULTS

Both mutants and wt behaved in the mixed cultures (Fig. 1) as they did when cultured alone (8, 9). In addition, the results confirmed the validity of the ratio method that we proposed to use for the in vivo experiments. The duplication time was 26 min for *E. coli*, whether calculated in the usual way from the absolute numbers of CFU (Fig. 1b) or when determined by the declining ratio method (Fig. 1a). The values for *P. aeruginosa*, 31 and 28 min, respectively, were not significantly different (Fig. 1c and d).

The results of two of several experiments with mixed ts-wt inocula of *E. coli* and *P. aeruginosa* in the peritoneal cavities of mice are shown in Fig. 2. The ratio of ts E/2/64 to wt *E. coli* began to fall immediately, but a slight lag occurred

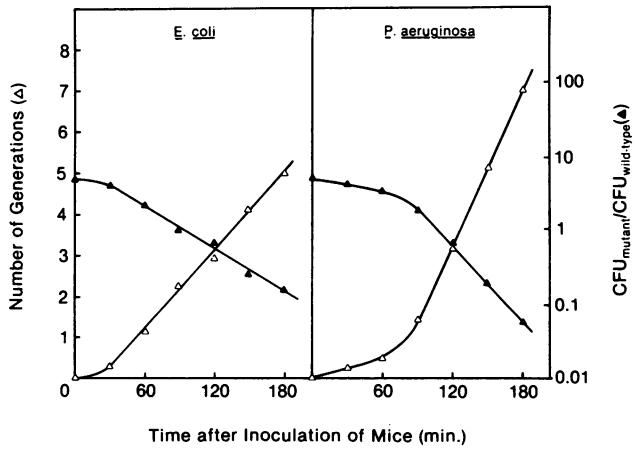


FIG. 2. Replication of *E. coli* and *P. aeruginosa* in the peritoneal cavities of mice. Symbols: (△) number of generations; (▲) ts/wt ratios. The MGTs for *E. coli* and *P. aeruginosa* were 33 and 20 min, respectively.

before the steepest rate of fall was achieved. The number of wt generations (from the formula above) was plotted versus time and showed a corresponding lag as the wt adjusted to the new environment before replicating at its maximum rate. The MGTs in the linear phase were 33 min for *E. coli* and 20 min for *P. aeruginosa*.

The sensitivity of the method is demonstrated in Fig. 3, in which we compared the replication in vivo of wt *P. aeruginosa* and a multiple-antibiotic-resistant derivative which, in vitro, has a duplication rate significantly slower than that of the wt. The in vivo rate followed the same pattern.

A series of experiments was performed with mixtures of the ts and either wt or the revertant of the ts mutant in the lungs of mice. The rationale for these experiments was that any subtle surface changes induced by mutagenesis and affecting surface recognition by the defense mechanisms of the host would be the same in both the ts mutant and its revertant. The MGT of the revertant of D/1/8 in the lungs (56 min), determined from the linear portion of the curves, equaled that of the wt (Fig. 4).

## DISCUSSION

The simultaneous clearance of bacteria in infected hosts prevents direct determination of in vivo replication rates. Several investigators have developed methods for measuring in vivo replication. In 1960, Hart and Rees compared the total numbers of *Mycobacterium tuberculosis* in the lungs of mice (determined by fixing, staining, and microscopic enumeration of organisms in tissue homogenates) with the numbers of viable cells (3). This method is not, however, generally applicable to other microorganisms because it depends upon the retention of acid-fast staining properties by dead *M. tuberculosis*.

Meynell used an ingenious system of lysogenized *E. coli* superinfected with a mutant of phage lambda but was unable to detect any replication of the organism in the spleens of mice (6). He then extended this idea to *Salmonella typhimurium*, using both lysogenized bacteria superinfected

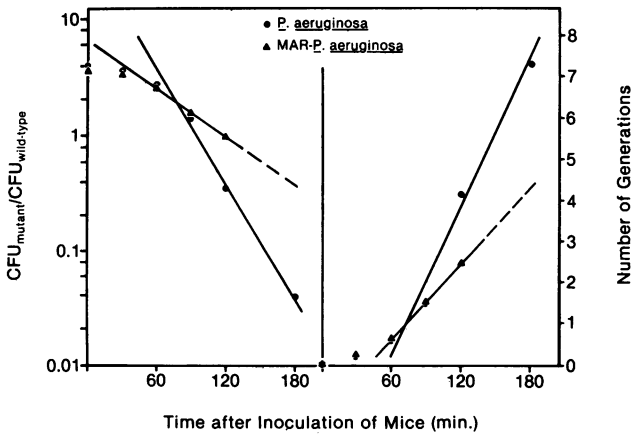


FIG. 3. Comparison of the in vivo replication rates of *P. aeruginosa* and a multiple-antibiotic-resistant (MAR) derivative. Left panel, ts/wt ratios plotted versus time. Right panel, Number of generations of wt versus time. MGTs were calculated from the slopes of the regression lines of the linear portion of the curves. The MGT for multiple-antibiotic-resistant *P. aeruginosa* was 43 min, and the MGT for *P. aeruginosa* was 20 min.

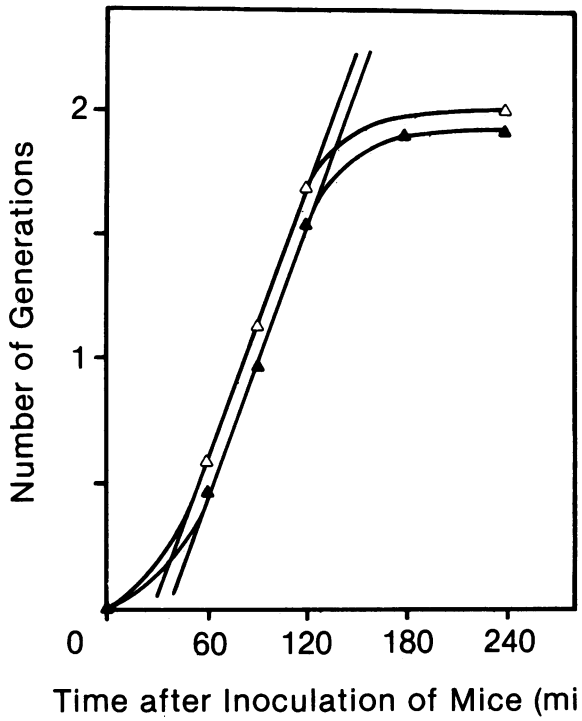


FIG. 4. Comparison of growth rates of *P. aeruginosa* and a revertant of the ts mutant D/1/8 in the lungs of mice. The number of generations is plotted as a function of time after aerosol inoculation. The MGTs were calculated from the slopes of the regression lines of the linear portion of the curves and were 56 min for both strains. Symbols: (△) revertant; (▲) wt.

with a mutant of P22 and *S. typhimurium* abortively transduced to streptomycin resistance (5, 7). The superinfection system was further refined and utilized by Hormaeche to study the mechanisms of natural resistance to *S. typhimurium* in genetically resistant and genetically susceptible mice (4). This technique, however, is restricted to those organisms with well-characterized lysogenic and mutant phages. It is also somewhat cumbersome in that the enumeration of the replicating and nonreplicating fractions requires the induction and detection of two different phages on two different indicator strains. In addition, even under the best conditions defined by Hormaeche, the nonreplicating component of the ratio always begins at less than 1, reducing the sensitivity of the method after several generations. It should also be noted that Meynell and Hormaeche both reported relatively high MGTs for *S. typhimurium* in the spleens of mice (3 to 12 h). However, these were measured over days, and few determinations were made in the first hours after inoculation.

Radiolabeled bacteria have been used to measure in vivo replication. Baselski, Medina, and Parker reported in 1978 that virulent *Vibrio cholerae* multiplied in the upper bowels of infant mice at the rate of 1.5 generations in 4 h (1). These authors also discussed at length the problems that might be encountered when using radioisotopes to measure in vivo MGTs and cautioned readers on the difficulties of interpreting decreases in specific activity (counts per minute/CFU), which could be due to leakage of the label and subsequent removal of soluble fractions away from the area being sampled. Sigel, Finkelstein, and Parker (10), using the same

method, compared the doubling times of another virulent strain of *V. cholerae*, 3083, and its nontoxigenic mutant, Texas Star-SR, in the infant mouse model and estimated MGTs of 60 and 65 min, respectively. Finally, Freter and O'Brien evaluated the use of different radioisotopes in experiments designed to measure in vivo replication, and their conclusions cast "considerable doubt on the general validity" of this method (2).

In the model proposed here, the interpretation of *n* as the number of generations of wt in the in vivo experiments rests upon two assumptions, i.e., that the ts mutant does not replicate at body temperature and that both the ts and wt are cleared by the animal at the same rate.

Although the ts mutants do not replicate at 36°C or above in vitro in rich media (Fig. 1), including serum (9), we have no direct proof that replication does not occur in vivo. We can, however, infer from the raw data of the CFU per milliliter recovered from the peritoneal lavages that any replication of the ts mutants is below the limit of detection and would have insignificant impact on the measurement of wt replication rates. The strongest evidence that D/1/8 does not replicate in vivo is that we have used mixtures of this mutant (carrying an antibiotic resistance marker) and other ts mutants of *P. aeruginosa* that undergo two or five duplications at body temperature to confirm the degree and rate of residual replication of these "coasters" in vivo (A. Morris Hooke, D. O. Sordelli, M. C. Cerquetti, and J. A. Bellanti, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B150, p. 42). Since the rate at which the coasters replicate at the nonpermissive temperature is very slow (MGTs = 70 to 110 min), significant replication of D/1/8 would almost certainly prevent the detection of coasting in vivo, let alone accurate determination of rates.

Indirect evidence for the validity of the second assumption, that the ts and wt strains are being cleared by animals at the same rate, was obtained in the experiments with mixtures of the ts *P. aeruginosa* mutant and either its revertant or the wt. Mutagenesis-induced alteration of surface molecules involved in interactions with complement components or phagocytic cell receptors could impair the clearance of the ts mutant, leading to falsely high values for the MGT of the wt. The revertant, however, would have the same surface molecules as the ts mutant; therefore, clearance mechanisms dependent upon intact ligands would act equally on the two strains. The MGTs for the revertant and the wt were identical (Fig. 4). If the receptors of the ts mutant were in fact altered, then the MGT of the revertant should have been lower. Other published data from our laboratory showed that the in vitro uptake and killing of *E. coli* mutant E/2/64 and its parental wt by polymorphonuclear leukocytes are similar (9).

The susceptibility of replicating microorganisms to the bactericidal mechanisms of the host may be different from that of nonreplicating bacteria. If the replicating cells are more susceptible, then the method of ratios employed here would yield slower measured replication rates and increased MGTs for the wt. The higher MGT obtained for *P. aeruginosa* in mouse lungs (56 min [Fig. 4] versus 20 min in the peritoneal cavity [Fig. 2]) might be due to increased susceptibility of the wt, although a more likely explanation is that the MGTs are actually different in the two areas of the body since they would clearly depend upon a variety of factors (e.g., $pO_2$, pH, and availability of nutrients). The changes in the slope of *n* versus time observed 3 h after aerosol exposure probably reflect the loss of sensitivity of the method due to the rapid clearance of both mutant and wt. If, on the other hand, the replicating wt is less susceptible, then the ts /wt ratios would decline even more quickly than they appear to, leading to physiologically improbable values for the calculated MGT of the wt.

The studies presented here offer a new approach for determining quantitative replication rates in vivo. This investigation was performed with ts mutants isolated after chemical mutagenesis. It would, however, be preferable to use spontaneous ts mutants for studies of this kind to avoid any possibility that other mutations could influence the system.

One of the most important advantages of this method is that the use of mixed inocula and the subsequent determination of ratios almost completely avoid the wide dispersion of data invariably encountered when one must rely upon the averages of absolute CFU values derived from individual animals.

The utilization of ts mutants that are unable to replicate at body temperature should be applicable to almost any pathogen of interest and could result in a greater understanding of the role of replication in pathogenic mechanisms and, subsequently, a more informed basis for antimicrobial strategies.

## ACKNOWLEDGMENTS

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