breeding of axenic animals (5). For this reason, a large body of data on the efficiency of the decontamination and air filtration systems is available. The data show excellent efficiency, at least as far as bacterial contamination is concerned (6).

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8 February 1977

Degradation of DNA by Nucleases in Intestinal Tract of Rats

Abstract. *Strains of* Escherichia coli *K12 have been constructed as safer hosts for use in recombinant DNA research. These strains are unable to survive passage through the intestinal tracts of rats because of a constellation of mutations conferring bile sensitivity and requirements for diaminopimelic acid and thymine. Since death caused by diaminopimelic acid deprivation could release recombinant DNA before DNA is degraded because of thymine starvation, it is important to determine the "survival potential" of the released DNA's. Bacterial and plasmid DNA's extracted from bacterial cells are rapidly degraded when added to low dilutions of rat intestinal contents. This observation, coupled with the stringent requirements necessary for in vitro transformation or transfection, make in vivo transmission of naked recombinant DNA in the rat intestinal tract highly improbable.*

The design, construction, and testing of strains of *Escherichia coli* K12 that are more useful and safer for recombinant DNA molecule research is one of the goals of our laboratory (*1*). The first of such strains, χ1776, is unable to survive passage through the intestinal tracts of rats at frequencies of less than 10^{-11} bacteria per milliliter because of a constellation of mutations that cause sensitivity to bile and requirements for diaminopimelic acid (DAP) and thymine (*1*). Although DNA degradation accompanies thymine-less death in in vitro experiments and is an all-or-none response for any given cell (*1*), it is likely that lysis associated with DAP-less death may liberate DNA prior to its degradation. Since undegraded recombinant DNA might be taken up by robust microorganisms encountered in natural environments by transformation or as a consequence of concomitant phage infection (*2*), it is important to determine the "survival potential" of such DNA in nonlaboratory controlled environments. We determined the rate of degradation of *E. coli* chromosomal and plasmid DNA in extracts of the intestine contents of rats. We were unable to locate any literature reports indicating the fate of "naked" DNA's in the intestines of warm-blooded animals and believed that a likely route of escape of chimeric microorganisms from the laboratory is ingestion by laboratory workers.

The small and large intestines were aseptically removed from asphyxiated rats (Charles River) (*3*), and after longitudinal incisions were made the contents were removed by washing with 5.0 ml of buffered saline with gelatin (BSG) (*4*) containing 10 m*M* $MgSO_4$. Each extract had a volume of approximately 6.0 ml and thus was a 1 : 6 dilution of the original intestine contents. The data in Fig. 1 show that there is an initial rapid degradation of the DNA in the 1 : 6, 1 : 12, and 1 : 60 dilutions of the small intestine extracts while no detectable degradation is observable in the large intestine extracts. After 4 hours, the level of DNA degradation in the diluted large intestine contents is comparable to the initial level of degradation in the small intestine contents, while complete degradation occurs in the 1 : 6, 1 : 12, and 1 : 60 dilutions of the small intestine contents. There was no DNA degradation during the experiment in the boiled extracts and controls containing BSG and 10 m*M* $MgSO_4$. In order to ascertain if the immediate reduction in acid-insoluble radioactivity in the diluted small intestine contents was due to quenching, the total soluble and insoluble radioactivity was monitored throughout the experiment and found to be equal to that in the input DNA. Results similar to those in Fig. 1 were obtained in another experiment in which the intestine contents were diluted in BSG without added $MgSO_4$. Thus a suitable ionic environment exists within the intestine for nuclease activity and our results are not enhanced by the addition of Mg^{2+} to the buffer used for suspending and diluting intestine contents. Comparable but somewhat more extensive DNA degradation occurs in the intestine contents from gnotobiotic rats (Fig. 2). These results rule out bacterial nucleases as being the sole cause for DNA degradation in the intestine contents from conventional rats. It therefore appears that the intestine contents of rats contain nucleases that rapidly degrade free bacterial DNA and are probably secreted into the lumen of the intestine by cells in the intestinal wall (*5*) or from the pancreas (*6*).

Data on the rate of bacterial DNA deg-

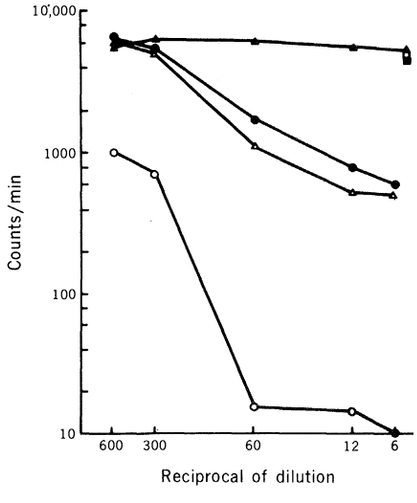

Fig. 1. Degradation of bacterial DNA by intestine contents from conventional rats. DNA labeled with tritiated thymidine was isolated from *E. coli* χ1776 (*1*). Lysozyme-generated spheroplasts were made in buffer containing 50 m*M* tris, 50 m*M* EDTA, 50 m*M* NaCl, and 10 percent sucrose (*p*H 8.0) and were lysed by adding 0.2 ml of spheroplasts to 1.0 ml of the intestine contents that were diluted to the extent indicated in BSG plus 10 m*M* $MgSO_4$. This method was believed to most closely mimic the in vivo entry of an enfeebled host into the lumen of the intestine. All incubations were at 37°C. Samples of 25 μl were taken immediately after mixing (0 hours) and at 4 hours and spotted on Whatman No. 3 filters. The filters were immersed sequentially in 10 and 5 percent TCA and 95 percent ethanol by standard methods and dried. The acid-insoluble counts were determined in a Beckman scintillation counter with 2,5-bis-2(5-*tert*-butylbenzoxazolyl)thiophene (BBOT) in toluene as a scintillation fluid. Data are shown for small intestine contents at 0 hours (●) and after 4 hours (○); large intestine contents at 0 hours (▲) and after 4 hours (△); small intestine contents boiled for 10 minutes before adding DNA at both 0 and 4 hours (■); and large intestine contents boiled for 10 minutes before adding DNA at both 0 and 4 hours (□). The control was DNA diluted into BSG plus 10 m*M* $MgSO_4$; its radioactivity remained at approximately 6200 count/min at 0 and 4 hours.

radation by small intestine contents from conventional rats are illustrated in Fig. 3. The 1 : 240 dilution of the small intestine contents was the lowest dilution that did not give DNA degradation in the time it takes to add the DNA and then sample the intestine contents. The rate of degradation in the 1 : 240 dilution is in good agreement with the degradation in the 1 : 300 dilution after 4 hours (Fig. 1).

The degradation of pMB9 DNA in 1 : 6 and 1 : 12 dilutions of small intestine contents from a conventional rat was qualitatively similar to that obtained with chromosomal DNA (Fig. 1); however, complete degradation of the plasmid DNA was achieved more rapidly (data not shown).

The data in Fig. 4 show the rate of degradation of plasmid pMB9 by small intestine contents from conventional rats. In order to properly evaluate this data it is necessary to attempt to relate the disappearance of trichloroacetic acid (TCA) insoluble counts to the number of surviving intact pMB9 molecules. The instantaneous rate of degradation of plasmid DNA is initially 24 min^{-1} and decreases to 0.48 min^{-1}. The decrease in rate probably reflects an inhibition of enzyme activity due to the generation of small polynucleotide fragments. The rate of inactivation of the plasmid, in terms of biological function, is much higher since only the action of endonuclease cuts is required to render the molecule linear and thereby lower its efficiency of transformation.

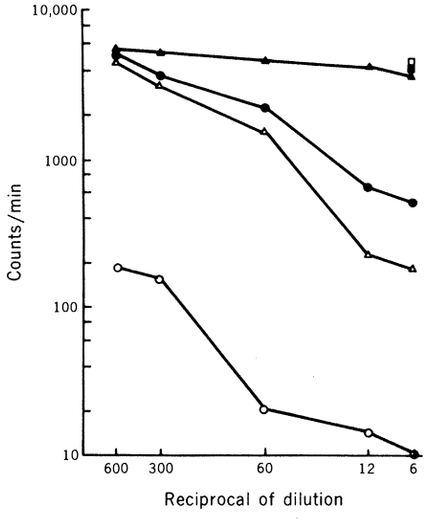

Fig. 2. Degradation of bacterial DNA by intestine contents from gnotobiotic rats. Methods were as described in the legend to Fig. 1. The intestine contents were found to be sterile. Data are for small intestine contents at 0 hours (●) and after 4 hours (○); large intestine contents at 0 hours (▲) and after 4 hours (△); small intestine contents boiled for 10 minutes before adding DNA at both 0 and 4 hours (■); and large intestine contents boiled for 10 minutes before adding DNA at both 0 and 4 hours (□). Radioactivity of the control, DNA diluted into BSG plus 10 m*M* $MgSO_4$, remained at approximately 4800 count/min at 0 and 4 hours.

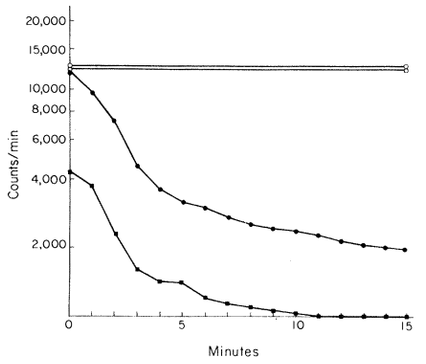

Fig. 3. Rate of degradation of bacterial DNA by small intestine contents from conventional rats. Methods were as described in the legend to Fig. 1. Data are for a 1 : 60 dilution of small intestine contents (■); a 1 : 60 dilution of boiled small intestine contents (□); a 1 : 240 dilution of small intestine contents (●); and a 1 : 240 dilution of boiled small intestine contents (○). Radioactivity of the control, DNA diluted into BSG plus 10 m*M* $MgSO_4$, remained at approximately 12,400 count/min at 0 and 15 minutes.

From our results, it is evident that a (gratuitous) safety feature exists in the rat intestinal tract that aids in rapidly degrading potentially hazardous DNA. We are concerned with the "survival potential" of the chimeric DNA in the small and large intestines because that is where the robust bacteria exist ($\sim 10^3$ bacteria per milliliter in the former and $\sim 10^{12}$ bacteria per milliliter in the latter) that could possibly be transformed by the chimeric DNA. Although we have not determined the actual site in the alimentary canal where χ1776 dies, we surmise that considerable killing would occur in the stomach due to gastric acid and in the duodenum at or after the juncture with the bile duct. If death of χ1776 in the small intestine is associated with cell lysis and release of DNA, then such DNA would immediately be exposed to pancreatic secretions, a most likely source of the intestinal nucleases, since the pancreatic and bile ducts are either joined together or adjacent in entering the duodenum (*7*). If some χ1776 cells survive passage through the small intestine and then lyse because of DAP-less death with release of DNA in the large intestine, this DNA would not be degraded as rapidly as in the small intestine (Figs. 1 and 2). However, the conditions that seem to be necessary for in vitro transformation of enteric bacteria with phage and plasmid DNA (*8*) (that is, cold $CaCl_2$ treatment of bacteria followed by a heat shock) are not likely to be found in nature and certainly not in the intestines of warm-blooded animals; this further reduces the probability that recombinant DNA released from phage or bacterial cells would be perpetuated in robust microorganisms in the intestine. Alternatively, DNA might be taken up concomitantly with phage infection (*2*). Although there is no readily available quantitative data on the titer of free phage in the intestines and no information on the ability of this mechanism to facilitate uptake of chimeric plasmid DNA, the rates of DNA degradation observed would seem to make such a mechanism of in vivo transmission improbable. Based on our data and the above considerations, we are of the opinion that death of disabled hosts such as χ1776 in the intestine will not lead to transmission of recombinant DNA to other enteric bacteria. The only qualification to this conclusion involves the question of whether the intestine contents of recombinant DNA molecule researchers, who are most likely to inadvertently ingest organisms containing recombinant DNA, are comparable to those of rats. However, should the pancreas be the major source of nucleases, it might be suggested that all recombinant DNA molecule researchers eat heartily before undertaking their experiments,

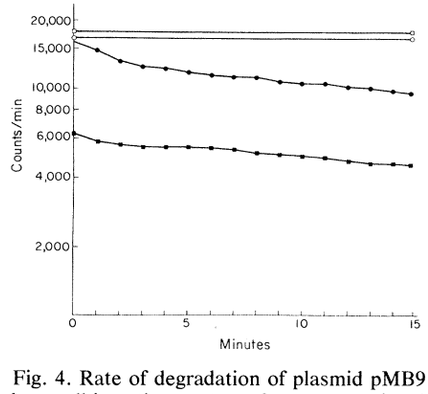

Fig. 4. Rate of degradation of plasmid pMB9 by small intestine contents from conventional rats. Covalently closed circles of the plasmid pMB9 were isolated in a cesium chloride–ethidium bromide gradient (*7*). The plasmid DNA was added to the intestine contents that were diluted to the extent indicated in BSG plus 10 m*M* $MgSO_4$. Methods were as described in the legend to Fig. 1. Data are for a 1 : 60 dilution of small intestine contents (■); 1 : 60 dilution of boiled small intestine contents (□); a 1 : 240 dilution of small intestine contents (●); and a 1 : 240 dilution of boiled small intestine contents (○). The control, plasmid DNA diluted into BSG plus 10 m*M* $MgSO_4$, remained at approximately 16,900 count/min at 0 and 15 minutes.

since feeding in normal humans stimulates both pancreatic and bile secretions into the duodenum (*7*). This last suggestion, although somewhat contrary to the advice given in the National Institutes of Health recombinant DNA research guidelines, may prove to be a safer practice when conducting recombinant DNA research with disabled hosts such as χ1776.

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9. Lysozyme spheroplasts were lysed with 0.1 percent Sarkosyl, heated 10 minutes at 68°C and mixed with cesium chloride–ethidium bromide (density 1.576) and centrifuged to equilibrium. Isopropanol was used to remove ethidium bromide; the dialysis buffer contained 20 m*M* tris, 2 m*M* EDTA, and 10 m*M* NaCl, *p*H 8.4.
10. The rats were provided by J. McGhee. Supported by NSF grant GB-37546 and NIH grants AI-11456 and DE-02670.

3 February 1977

Probability of Establishing Chimeric Plasmids in Natural Populations of Bacteria

Abstract. *Formulas for estimating the probability that chimeric plasmids carried by disarmed hosts will become established in natural populations of bacteria are presented and their use illustrated with a series of realistic numerical examples. The implications of these a priori probability estimates for the problem of containment for recombinant DNA research is discussed.*

At the very best, physical containment will retard the rate at which the bacteria, phage, and plasmids used in recombinant DNA research enter the environment. Some, and potentially great numbers, of these cloning vectors will escape. However, escape from containment does not necessarily imply that these renegade organisms and plasmids or their chimeric DNA will become established in natural populations of bacteria, or that carriers of this synthetic genetic material will achieve and maintain sufficient densities to represent a threat to existing communities. This contamination of natural populations of bacteria with chimeric DNA ultimately depends on the population biology of the cloning vectors. In recognition of the need for biological containment, "disarmed" strains of bacteria with low fitness have been developed, and phage and plasmids with limited infectious capabilities have been identified for use in recombinant DNA research (*1*). Although there is no question that employing the recommended cloning vectors offers a much lower likelihood of contaminating natural populations with chimeric DNA than that associated with the use of normal laboratory strains, just what this contamination probability is remains uncertain. We believe that there are two difficulties with the estimates of it that have been offered (*1, 2*): (i) failure of the estimation procedure to consider the population dynamics of the contamination process and (ii) inability to obtain accurate estimates of some of the parameters necessary to compute the contamination probability.

In this report we present a stochastic model of the establishment of a plasmid carried by a disarmed host into a natural population of bacteria. We do consider the population dynamics of the contamination process and, we believe, reasonably accurate estimates of all the parameters of this model could be obtained. Although we restrict this formal consideration to conjugative factors, the theory is appropriate to certain situations where nonconjugative plasmids can be mobilized (*3*). We present formulas for the probability of establishment of a chimeric plasmid and illustrate their use with a series of realistic numerical examples.

In this treatment we define establishment as the indefinite persistence of the chimeric plasmid in a natural population of bacteria. That is, bacteria carrying that plasmid will continue to exist and increase in numbers after all of the original, disarmed, hosts are eliminated. What densities "wild" bacteria carrying that plasmid will maintain and whether these factors will persist if the plasmid reduces the fitness of its host cell have been considered elsewhere (*4*).

Consider the release of m_0 disarmed, plasmid-bearing bacteria into a natural population containing r (bacteria per milliliter) potential hosts of that plasmid. The original hosts are incapable of growth in the natural habitat and, in the course of time, die or effectively die by losing the plasmid (segregation) or the capacity to transmit it. Let the probability of any particular disarmed plasmid-carrying bacterium dying or becoming infertile during the short period δt be $d\delta t$. We assume that all the wild bacteria have the same rates of cell division and cell mortality and that these rates are not influenced by carrying the chimeric plasmid. Let the probabilities of a particular wild bacterium dividing or dying during the period δt be $b\delta t$ and $e\delta t$, respectively. We further assume that transmission of the plasmid by conjugation occurs at random at a frequency that is proportional to the concentration of potential recipients. We let the probability that a single disarmed host will transmit the plasmid during time δt be $g\delta t = \gamma_d r\delta t$, where γ_d (milliliters per cell per hour) is the conjugational transfer rate constant (*4*). The analogous transmission probability for a single wild bacterium carrying that factor is $h\delta t = \gamma_h r\delta t$.

With the above definitions and assumptions, the establishment of the plasmid can be regarded as a population process (*5*) to be analyzed by means of generating functions. Using this approach, and after some relatively straightforward, albeit cumbersome, calculations, we concluded that the probability that the plasmid will be lost after the escape of a *single*, disarmed plasmid-bearing cell is

$$P_{loss} = \frac{d(b + h)}{d(b + h) + g(b + h - e)} \quad (1)$$

and hence the probability that the plasmid will become established is

$$P_{est} = 1 - P_{loss} = \frac{g(b + h - e)}{d(b + h) + g(b + h - e)} \quad (2)$$

The calculations that formally prove these results can be obtained from (*6*). In this report we restrict the justification of this probability estimator to an intuitive argument.

With biologically realistic values of the