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Long-Term Starvation-Induced Loss of Antibiotic Resistance in Bacteria

R. P. Griffiths,[1]* C. L. Moyer,[1] B. A. Caldwell,[1]
C. Ye,[1] and R. Y. Morita[1,2]

[1]Department of Microbiology, College of Science and [2]College of Oceanography, Oregon State University, Corvallis, Oregon 97331-3804, USA

Abstract. *Escherichia coli, Pseudomonas fluorescens,* and a *Pseudomonas* sp. strain 133B containing the pSa plasmid were starved in well water for up to 523 days. There were two patterns of apparent antibiotic resistance loss observed. In *Pseudomonas* sp. strain 133B, there was no apparent loss of antibiotic resistance even after starvation for 340 days. In *E. coli,* by day 49 there was a ten-fold difference between the number of cells that would grow on antibiotic- and nonantibiotic-containing plates. However, over 76% of the cells that apparently lost their antibiotic resistance were able to express antibiotic resistance after first being resuscitated on non-selective media. By day 523, only 12% of these cells were able to express their antibiotic resistance after being resuscitated. After starvation for 49 days, cells that could not grow on antibiotic medium even after resuscitation, showed a permanent loss of chloramphenicol (Cm) resistance but retained resistance to kanamycin (Km) and streptomycin (Sm). Restriction enzyme digests show that a 2.5 to 3.0 Kb region from map location 12.5 to 15.5 Kb was deleted. This coincides with the 2.5 Kb reduction in plasmid size observed in 3 isolates that had lost antibiotic resistance after starvation for 49 days.

Introduction

The loss of gene expression under starvation conditions without the loss of genetic material has important implications in tracking genetically engineered microorganisms in natural ecosystems. If one is depending on using antibiotic selective plate counts to trace resistance markers in organisms inoculated into natural water or soil samples, the number of organisms recovered using this method could easily be greatly underestimated. It is also possible that antibiotic resistance markers associated with the plasmid may be permanently lost due to partial or complete degradation of plasmid DNA.

Devanas et al. [4] have reported that bacteria with plasmids containing an

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* Corresponding author.

antibiotic marker cannot initially grow on selective media after they had been added to nonsterile soils. This suggests that the cells were so debilitated from starvation that they had temporarily lost their antibiotic resistance. However, when these cells were initially isolated on a nonselective medium and then transferred to selective medium, they again exhibited their antibiotic resistance. This phenomenon has also been documented in a number of host strains containing different plasmids with drug resistance markers [2]. Cells of *Escherichia coli, Enterobacter agglomerans,* and *Klebsiella pneumoniae* containing the plasmid R388::Tn1721 that were starved in well water had over 90% of the population demonstrate temporary loss of antibiotic resistance expression after less than 50 days starvation. Essentially the entire population temporarily lost antibiotic resistance after starvation for 150 days. In all instances, however, over 95% of the population recovered antibiotic resistance after being resuscitated on Luria-Bertani (LB) plates [2].

It is suspected that, when the cells are starved, the enzymes required to detoxify the antibiotic are no longer present. These cells still retain the plasmid DNA that codes for antibiotic resistance, but under these conditions the proteins are either absent or are inactive. When these cells are resuscitated by exposure to a complete medium without the antibiotics, they are able to replace these resistance-mediating proteins that were lost during starvation.

These observations strengthen the idea that plasmid-containing bacteria can survive for long periods of time even though they might not be recoverable using standard plating methods on selective media. Genetically manipulated plasmids contained within bacteria that have been grown under laboratory conditions and then inoculated into natural samples have shown the ability to survive for extended periods up to 72 days [3, 4, 7].

The above studies are those in which entire plasmids or plasmid function have been lost due to a number of factors. There have been relatively few studies in which a partial loss of plasmid DNA function has been documented in response to environmental stress factors. In batch culture growth studies, pSa in *Zymomonas mobilis* was shown by Strzelecki and coworkers [9] to lose chloramphenicol (Cm) resistance in 10% of the isolates grown on selective medium containing kanamycin (Km) or spectinomycin (Sp), whereas both Km and Sp resistance remained [9]. They reported that the loss of Cm resistance was associated with a reduction in plasmid size. Caldwell et al. [2] reported a reduction in the size of plasmid R388::Tn1721 in *Klebsiella pneumoniae* starved cells which was accompanied by loss of antibiotic resistance. The main objective of the current study was to define which portion of a plasmid is lost during starvation. To accomplish this, starvation patterns of different strains containing the pSa plasmid were documented, along with an analysis of where along the plasmid map there was a deletion which caused the loss of function observed in *E. coli* as the result of starvation.

Materials and Methods

The bacterial strains used in this study were *E. coli* strain 100 and a *Pseudomonas* sp. strain 133B, obtained from M. Walters at the Corvallis EPA laboratory, and *P. fluorescens* containing plasmid

pSa, which was obtained by conjugation of *P. fluorescens* obtained from J. Loper of the U.S.D.A. Agricultural Research Service, Corvallis, OR. Log phase cells were grown in 100 ml Luria-Bertani broth (LB) [8] containing 25 μg/ml chloramphenicol (Cm), 25 μg/ml streptomycin (Sm), and 50 μg/ml kanamycin (Km) at 30°C on a rotary shaker at 200 rpm for 18–24 hours. All antibiotics were purchased from Sigma Chemical Co., St. Louis, MO. The cells were harvested by centrifugation at 6500 × *g* for 10 minutes at 20–27°C. The pellet was resuspended in 100 ml of sterile well water. Portions of the resuspended culture were added to a 1-liter Wheaton bottle containing approximately 800 ml autoclaved well water until an optical density of approximately 0.02 (A_{600}) was reached, yielding a final cell suspension concentration of approximately 10^7 colony forming units (CFU)/ml. A sterile teflon-coated magnetic stir bar was added to uniformly mix the cell suspension prior to sampling.

Starvation menstruums were incubated at ambient temperatures, and cell populations were periodically monitored by serial dilution onto selective (containing appropriate antibiotics) and nonselective (containing no antibiotics) LB agar plates. Plasmid expression is defined as the portion of organisms initially growing on selective medium relative to the total number of viable organisms recoverable on nonselective media. Those organisms that could not initially grow on the selective medium were those that temporarily or permanently lost expression of the antibiotic resistance. To determine which portion of this population had permanently lost resistance, 100 colonies were restreaked from the nonselective plates onto either a selective plate containing all three antibiotics or three selective plates containing either Km, Sm, or Cm. The restreaked plates were incubated at 30°C for 1–4 days. The percentage of colonies maintaining antibiotic resistance upon resuscitation was calculated after counting the number of colonies that grew upon restreaking.

Sterile well water, adjusted to pH 7.0 ± 0.3, was used in preparing starvation cell suspensions and sample dilutions. Sterilization of media, well water, reagents and equipment was by autoclaving. Triplicate LB agar plates with and without Cm, Sm, or Km were used to measure concentrations of total and antibiotic resistant cells. The following concentrations of antibiotics were used: Cm, 25 μg/ml; Sm, 25 μg/ml; Km, 50 μg/ml. Antibiotics were sterilized by filtration through 0.20 μM disposable 25 mm filter assemblies (Acrodisc, Gelman Sciences, Ann Arbor, MI).

Bacterial isolates to be used for plasmid analysis were grown in 10 ml of LB medium for approximately 24 hours and purified by alkaline-lysis minipreparation [1]. Purified plasmids were digested with the restriction enzymes: *Sma*I, *Eco*RI, *Bgl*II, *Bam*HI, *Hind*III, and *SST*II which were all purchased from Life Technologies, Inc., Gaithersburg, MD (BRL). Gels containing 0.7% agarose (BRL electrophoresis grade) and 0.5 μg/ml ethidium bromide (EtBr) were viewed on a transilluminator at a wavelength of 260 nm. Gels were photographed with a Polaroid 107 pack film (Polaroid, Inc., Hertfordshire, England). Sample DNA from gels was blotted onto a membrane filter (Gene Screen$^+$, NEN; Research Products; DuPont, Co., Boston, MA) using the VacuBlot-VS apparatus from American Bionetics, Inc. (Hayward, CA).

Plasmid DNA for the preparation of the hydridization probe was extracted by a large-scale alkaline-lysis plasmid preparation and then isolated by cesium chloride (CsCl)-EtBr equilibrium density gradient [1]. The pSa DNA was digested with *Sma*I and further purified by gel filtration onto NA-45 DEAE-cellulose membranes (Schleicher and Schuell, Keene, NH). Plasmid DNA was then nick translated (BRL Nick Translation System) with biotin-7-dATP and purified with IBI Nu-Clean D50 columns (International Biotechnologies, Inc., New Haven, CT). Biotinylated plasmid single-stranded DNA was hybridized to Vacusystems blots utilizing the BRL BluGENE Nonradioactive Nuclei Acid Detection System.

Results and Discussion

E. coli strain 100N, *P. fluorescens* (strain 411A), and *Pseudomonas* sp. strain 133B species, each containing pSa, were starved in sterile well water for up to 523 days. In *E. coli*, a loss in antibiotic resistance was observed within the first 50 days of starvation (Fig. 1A). Although there was no significant loss in apparent antibiotic resistance during the first 12 days of starvation, after 49

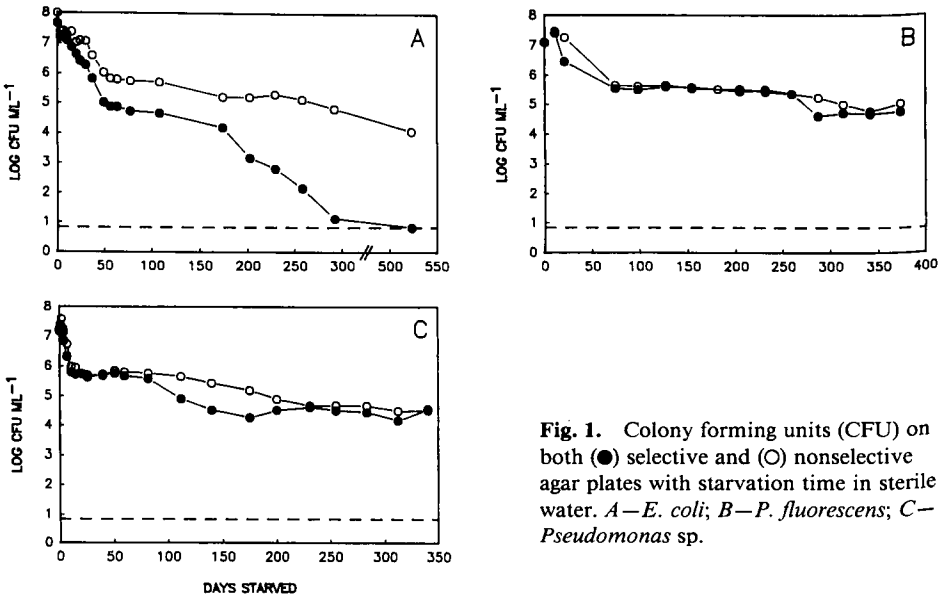

Fig. 1. Colony forming units (CFU) on both (●) selective and (○) nonselective agar plates with starvation time in sterile water. *A—E. coli*; *B—P. fluorescens*; *C—Pseudomonas* sp.

days only 10% of the total viable population (those growing on nonselective agar plates) retained their antibiotic resistance on initial recovery. This pattern is in stark contrast to that observed in the two *Pseudomonas* strains (Fig. 1B, 1C).

To measure the proportion of the *E. coli* population still retaining functional plasmids, 100 isolates were screened on LB plates containing Km, Sm, or Cm after initial growth on a nonselective medium. Of these isolates, 99% grew on Km or Sm and 96% grew on Cm. These results suggested that, although most of the isolates showed only a temporary loss of resistance during starvation, only 1 to 4 percent of the population had permanently lost resistance. Isolates that had permanently lost their ability to grow on selective media containing either Km, Sm, or Cm in response to starvation were screened for changes in plasmid size by analyzing plasmid bands on agarose gels. Of the 100 isolates analyzed, 3 showed a reduction in plasmid size, and in one, no plasmid could be detected. Plasmids from three of the isolates showing permanent loss of Cm resistance were digested with the restriction enzymes *Eco*RI and *Bam*HI yielding a pattern of banding that when compared to restriction enzyme maps, strongly suggested that the area from 12.5 to 15.5 Kb had been deleted (Fig. 2). The same pattern was observed in all three isolates.

Using restriction enzyme *Eco*RI, a 2.5 to 3.0 Kb section was missing from the starved culture (lane J, Fig. 2A) when compared to the nonstarved control (lane K, Fig. 2A). Both *Eco*RI (lanes J and K) and *Bgl*II (lanes H and I, Fig. 2A) fragments show the lower end of the deletion whereas the fragments from the *Sma*I (lanes L and M) define the upper end of the deletion. Similar results were observed in plasmids from starved and nonstarved cultures treated with the restriction enzymes *Hind*III (lanes D and E) and with *SST*II (lanes B and C). When plasmids from starved cells were digested with *Bam*HI (lanes F and

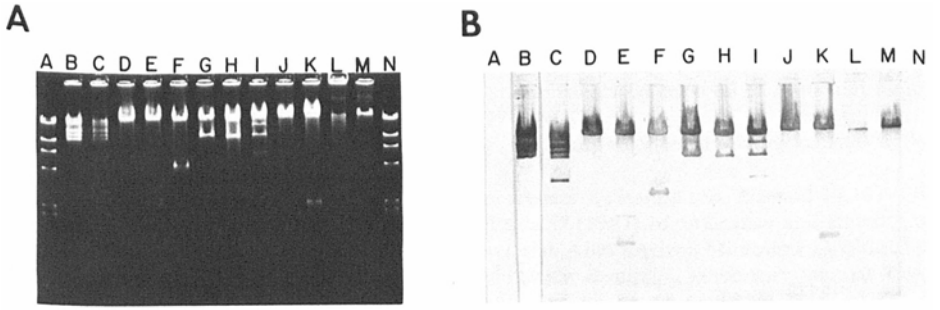

Fig. 2. Photograph of electrophoresis gel with lanes showing control and starved plasmids digested with restriction enzymes. *A*—Separation of digested plasmid DNA by agarose gel electrophoresis. *B*—Photograph of Southern blot hydridized with biotinylated pSa probe. The source of the plasmid material was: (A) lambda *Hind*III digest, (B) plasmid from starved cells digested with *SST*II, (C) plasmid from nonstarved cells digested with *SST*II, (D) starved cells with *Hind*III, (E) nonstarved cells with *Hind*III, (F) starved cells with *Bam*HI, (G) nonstarved cells with *Bam*HI, (H) starved cells with *Bgl*II, (I) nonstarved cells with *Bgl*II, (J) starved cells with *Eco*RI, (K) nonstarved cells with *Eco*RI, (L) starved cells with *Sma*I, (M) nonstarved cells with *Sma*I, and (N) lambda *Hind*III digest.

G in Fig. 2A), the bounds and size of the deletion can be determined. A hybridization analysis of a Southern blot of the digested segments indicates that all fractions present came from the original intact pSa plasmid (Fig. 2B).

Comparison of fragments from the restriction enzyme digests with published maps [6, 10] of pSa indicates that the fragment from about 12.5 to 15.5 Kb, which contains Cm resistance, is missing (Fig. 3). It has been suggested by Tait et al. [10] that spontaneous Cm resistance loss in pSa corresponded to the deletion of the 10.5 to 14.5 Kb section of the plasmid. This is very close to the 9.5 to 12.7 Kb section that, according to Ward and Grinsted [11], codes for Cm resistance.

One of the objectives of this study was to determine if the spontaneous loss of Cm resistance observed by Tait et al. [10] involved the deletion of the same section as that observed by us under starvation conditions. The above results suggest that they are essentially the same phenomenon. This is not however to suggest that this is the only deletion that can take place in this plasmid. Cells that had been starved for 523 days still showed a difference of four orders of magnitude between total CFU and the number that had lost antibiotic resistance upon plating directly from the cell suspension. When 100 of these cells were restreaked on Km, Cm, or Sm plates after resuscitation on a nonselective medium, only 12% were able to grow on any of the antibiotic plates.

There is currently a great deal of interest in monitoring of genetically engineered microorganisms (GEMs) released into the environment [5]. There are a number of obvious problems with tracking not only the organisms containing the altered DNA but also the DNA itself. Because of logistical constraints on tracking these organisms, metabolic genetic markers and differential plating schemes are usually employed in GEM tracking procedures. This study shows that such a marker might be lost from a population of laboratory produced GEMs when they are applied to a natural substrate that induces starvation

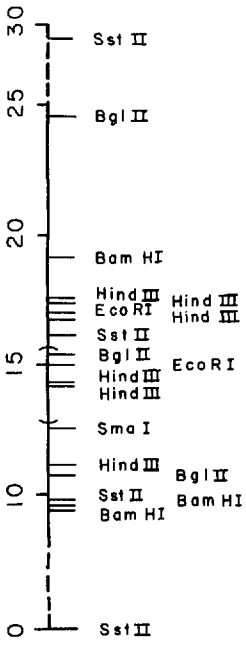

Fig. 3. A map of restriction enzyme sites and the location of the segment that was apparently lost during starvation. (The lost segment is enclosed within parentheses.)

survival conditions. In this case, the marker for Cm can be lost, but a portion of the plasmid genome can remain within the organism and it can continue to carry this plasmid for an extended period of time. We have also shown that the host organism can greatly influence plasmid genetic expression. If this plasmid had been used for tracking purposes in the study of *Pseudomonas* sp., there would be no apparent loss in plasmid expression. However, if the plasmid was placed into *E. coli* and released, standard selective medium tracking procedures would have greatly underestimated the number of organisms present that still contained that genetic material. From this we must conclude that starvation studies on GEMs should be conducted on the exact combination of host and genetic material that is to be released into the environment.

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