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Survival of Genetically Modified *Escherichia coli* Carrying Extraneous Antibiotic Resistance Gene through Microbial Interactions

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The advance of recombinant DNA techniques allows us to construct novel bacterial strains. The potential uses of such recombinant bacteria in the environment include biocontrol of insects, environmental remediation and waste treatment (Alexander 1981). Intended release of such genetically engineered microorganisms (GEMs) into natural environments has, however, raised questions regarding their ecological impact and many studies are being conducted to evaluate their ecological effects (e.g. Goodnow et al. 1990; Min et al. 1998; Tiedje et al. 1989). The safety of releasing GEMs is still uncertain and, in Japan, the field application of GEMs is prohibited by law to prevent possible ecological impact. Recently, much more environmental concern has been paid to the unintended release of laboratory bacterial strain, which may be recombinant, for research purposes. (Av-Gay 1999). Although we can regulate the deliberate release of GEMs into environment, we are still exposed to unintended release of recombinant bacteria.

Bacteria such as *Escherichia coli* K-12 are intensively used as laboratory microorganisms in the area of molecular biology and their genetic characteristics are frequently modified for research purposes by introducing a foreign gene. Using *E. coli* K-12 as the parental strain for genetic manipulation has been deemed safe because not only have their modified characteristics led to loss of their ability to survive in natural environments but they are also burdened with the increased metabolic demands of the maintenance and expression of the introduced foreign genes. Several studies have been conducted to evaluate the effects of releasing genetically modified *E. coli* K-12 into the environment (e.g. Chao and Feng 1990; Helling et al. 1981) but the results were inconsistent. Some support the safety of releasing them and some cast doubt on their safety (Brill 1985; Sobecky et al. 1992). To evaluate the impact of unintentionally releasing genetically modified *E. coli* K-12, it is necessary to focus on the factors, which are not site-specific, involved in its survival.

In this study, we focused on the effects of microbial interactions, which including the competition with the parental strain, and the existence of protozoa and algae, as factors which would be involved in the survival of genetically modified *E. coli* K-12. For this purpose, we used a species-defined microcosm, which consisted of the bacterium *E. coli*, a bacteria feeding protozoan *Tetrahymena thermophila* and a photosynthetic alga *Euglena gracilis* (Kawabata et al. 1995). In this system, *T.*

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thermophila interact with *E. coli* directly through predation but *E. gracilis* interact with *E. coli* only through its metabolites (Matsui et al. in press). Therefore, we will be able to evaluate both direct and indirect effects of these processes on the survival of the genetically modified *E. coli* K-12 in this system. We also tried to evaluate the impact of the genetically modified *E. coli* K-12 for the population dynamics of the protozoan and the alga in this microcosm.

MATERIALS AND METHODS

E. coli K-12 DH5α (Sambrook et al. 1989), designated here as *E. coli*, is a laboratory strain commonly used for molecular biological studies and is used here as the parental strain of the genetically modified strain. Plasmid pBluescriptII SK(+) (Alting-Mees et al. 1989), encoding the antibiotics ampicillin resistance gene, is a plasmid vector widely used for DNA recombination. It was transformed into *E. coli* to construct the genetically recombinant strain, harboring the extraneous plasmid, designated here as *E. coli* (P). *E. coli* and *E. coli* (P) were maintained on Luria-Bertani (LB) agar medium (Sambrook et al. 1989) and LB with 50μg/ml ampicillin agar medium respectively.

E. coli (P) was introduced into an *E. coli* single culture to test competition with the parental strain. Half strength #36 Taub's salt solution (Taub and Doller. 1968) without $NaNO_3$ was supplemented with 500 mg/L of proteose peptone (Difco laboratory, USA) and used as a culture medium. The medium was decanted into a series of test tubes and autoclaved before inoculation with the organisms. To synchronize the condition of the cells, *E. coli* were precultured in LB medium until they reached the late-logarithmic growth phase, washed twice in half strength Taub's #36 salt solution, resuspended in the same solution and then inoculated into each test tube at 10^2 cells/mL, the initial population density. Each test tube was placed stationary, in an incubator under 2500 lx by fluorescent lamps with 12-12LD light regime at 25°C and were sacrificed for counting on each sampling day. *E. coli*(P) was introduced into *E. coli* cultures at the beginning and after 5-days of the cultivation period. The numbers of *E. coli* (P) were monitored by colony formation units (CFU) on broth-agar plate medium (extracted bonito 3.0 g, polypeptone 3.0 g, NaCl 5.0 g, and 15 g agar powder in 1 liter of distilled water; pH adjusted to 7.0) with 0.5mg/L of ampicillin. The total cell number was obtained by the CFU on nutrient agar without ampicillin. Each count was carried out for three petridishes and the mean values of three counts were used as CFU per mL.

E. coli (P) were also introduced into a simple aquatic microcosm, which was developed by Kawabata et al. (1995), to evaluate the effects of microbial interactions on the survival of the recombinant strain. The microcosm consisted of a photosynthetic alga *Euglena gracilis* (producer), a bacteria-feeding protozoan *Tetrahymena thermophila* (consumer) and bacterium *E. coli* (decomposer) and was thus, employed to represent a simplified aquatic microbial ecosystem (Kawabata et al. 1995). High reproducibility of this microcosm suggested its reliability as a model of a microbial community (Matsui et al. in press). The microcosm was constructed by inoculating the *E. gracilis*, *T. thermophila* and *E. coli* into culture medium by the same methods as used for *E. coli* single-culture except that the pre-culture of *T. thermophila* and *E. gracilis* was conducted in nutrient rich media (for *T. thermophila*; proteose peptone 2.5 g, yeast extract 2.5 g and dextrose 10 g in 1 liter of distilled water; pH adjusted to 7.0: for *E. gracilis*;

tryptone 10 g, yeast extract 1 g, dextrose 10 g and vitamin B_{12} 100 μg in 1 liter of distilled water; pH adjusted to 3.0). *E. coli* (P) was inoculated into the microcosm at the beginning of cultivation (day-0) and after 5-days of cultivation. The methods used to obtain the numbers of *E. coli* (P) and the total cell numbers were as described above. The numbers of *T. thermophila* and *E. gracilis* cell were counted under a microscope according to Kawabata et al. (1995).

RESULTS AND DISCUSSION

Figure 1 shows the population changes of *E. coli* (P) in the *E. coli* single-culture system. No significant difference in growth was observed between *E. coli* and *E. coli* (P) when the latter was introduced to the *E. coli* culture at the beginning of cultivation (Fig. 1A). This indicates that *E. coli* (P) has the same growth potential as the parent *E. coli*. In contrast, *E. coli* (P) declined sharply when introduced into *E. coli* single-culture after 5-days of incubation (Fig. 1B). The disappearence of *E. coli* (P) from the culture may reflect an uncomfortable environment for its growth, such as a scarcity of nutrients because both strains have the same metabolite pathways and would compete for each others resources. Another possibility was accumulation of metabolites from *E. coli* which might negatively affect the growth for both *E. coli and E. coli* (P) (Matsui et al. in press). No significant difference was observed in the population density of the parental strain with the addition of *E. coli* (P). From these results in the *E. coli* single-culture system, it seems less possible that unintentionally released *E. coli* (P) survive at a release site, where its parental *E. coli* already existed, due to competition with the parental strain.

When *E. coli* (P) were introduced into the three species microcosm at the start of cultivation, they grew in the same manner as the parent *E. coli* (Fig. 2A). However, *E. coli* (P) were also able to maintain their population density over 20 days when introduced into a three species microcosm on day 5 of cultivation (Fig. 2B). The addition of *E. coli* (P) did not affect the population densities of the other species in the microcosm (Fig. 2).

It has been well documented that predation by protozoa is an important factor in reducing the population densities of bacteria in aquatic environments (Nakano et al. 1998) and this is supported by the decrease of the *E. coli* population densities from 10^7 cells/mL (in single culture) to 10^6 cells/mL (in three species microcosm). Here, we found additionally that the existence of algae and protozoa in the microcosm ameliorated the competition between *E. coli* and *E. coli* (P) and provided conditions which allowed the survival of *E. coli* (P) (Fig. 2B), which had disappeared from the five-day old *E. coli* single culture. The metabolites from algae and protozoa would ameliorate the self-toxicity of the *E. coli* metabolites and thus support the survival of *E. coli* (Matsui et al. in press). Although the exact mechanism by which *E. coli* (P) maintained its population in the three species microcosm is not clear. In a previous study, we found that, in addition to protozoan predation, a metabolite of the indigenous bacteria were factors affecting the decreases of both *E. coli* and *E. coli* (P) when introduced to a paddy field microcosm (Kawabata et al. 1998). The results in the present study suggest that a metabolite from the competitive *E. coli*, might be a stronger factor than protozoa in the decrease of the introduced genetically recombinant *E. coli* and that genetically recombined *E. coli* used in a laboratory have the possibility of surviving in aquatic environments through such microbial interaction.

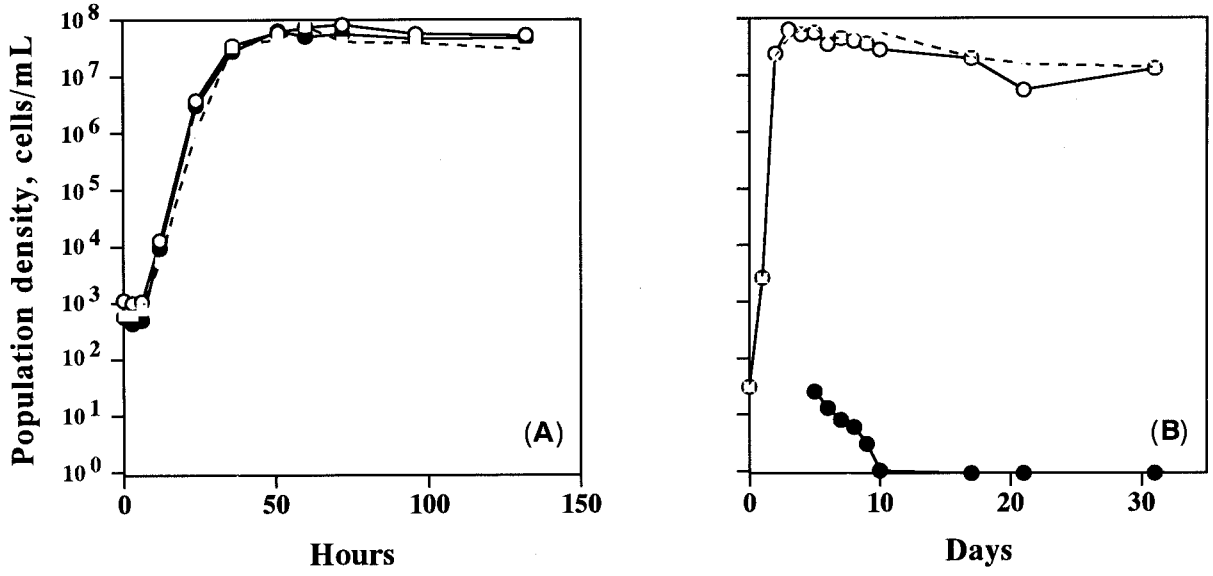

Figure 1. Population changes of *E. coli* (P)(●) introducing into *E. coli* single culture at the same time as *E. coli* (A), and after 5 days cultivation of *E. coli* (B). Population changes of *E. coli* (P) + *E. coli* (○), are also represented and broken lines indicate results of control experiments.

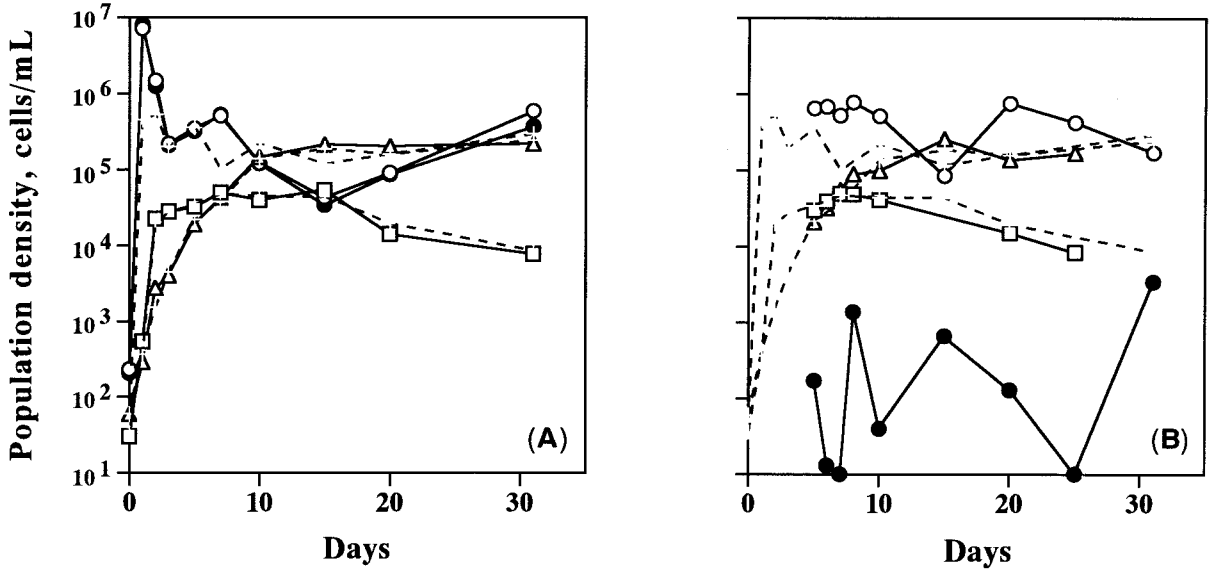

Figure 2. Population changes of *E. coli* (P)(●) introduced into a three-species microcosms at the same time as the other three species (A) and after 5 days cultivation of the other three species (B). Population changes of *E. coli* (P) + *E. coli* (○), *T. thermophila* (□), and *E. gracilis* (◇) are also represented. Broken lines represent results of control experiments.

The difference between intended and unintended release of GEMs are the genetic and physiological designs of the bacteria. With an intended environmental application the GEMs would often have as an objective their stable maintenance and function in a particular environmental niche. In contrast, the bacteria which were unintentionally released from a laboratory would originally be designed and recombined for a specific research purpose, such as cloning of a specific gene, and therefore, less attention would have been paid to its survival after experiments. In this study, we used the species defined microcosm and have demonstrated that the survival potential of a laboratory *E. coli* strain, through microbial interaction which may attenuate competition with the parental strain. Since there are several reports that *E. coli* is one component of the resident bacteria in aquatic environments (Brettar et al. 1992), our results indicate that the survival of laboratory used genetically recombined *E. coli* in aquatic environment is possible, where parental *E. coli* are resident.

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