BACTERIAL GRAZING-RESISTANCE DEVELOPED DURING CO-EXISTENCE WITH A BACTERIVOROUS PROTIST

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Two types of transformants with different drug resistance were developed from the phenol-oxidizing bacterium, *Cupriavidus* sp. KN1. The first transformant is *Cupriavidus* sp. KN1-TGF that has green fluorescence and tetracycline resistance. The second transformant is *Cupriavidus* sp. KN1-KRF that has red fluorescence and kanamycin resistance. These two strains can be selectively colonized with antibiotic-containing media, and they can be also distinguished by fluorescent color. First, *Cupriavidus* sp. KN1-TGF cells remaining after predation with the flagellate, *Spumella* sp. TGKK2, were mixed with *Cupriavidus* sp. KN1-KRF that did not contact with the flagellate TGKK2 to investigate the change in characteristics for the protistan predation. As a result, *Cupriavidus* sp. KN1-KRF was preferentially predated. On the other hand, *Cupriavidus* sp. KN1-TGF in the same tube was relatively resistant to predation. Similar phenomena were observed when the conditions of these two strains were reversed. Next, two complete mixing reactors connected in series were operated. Bacteria were cultured in the first reactor, and the protistan predation was made in the second reactor. Two runs were operated, one with *Cupriavidus* sp. KN1-TGF and the other with *Cupriavidus* sp. KN1-KRF. Then the first reactor effluent that contains *Cupriavidus* sp. KN1-KRF was mixed with the second reactor effluent that contains residual *Cupriavidus* sp. KN1-TGF and the flagellate TGKK2 in a tube. The cells of *Cupriavidus* sp. KN1-KRF not in contact with the protist were preferentially predated. This phenomenon was similarly confirmed by combinatorial inversion that *Cupriavidus* sp. KN1-TGF from the first reactor was mixed with *Cupriavidus* sp. KN1-KRF from the second reactor.

Key Words: protist, flagellate, grazing, resistance, prey, predator

1. INTRODUCTION

In the field of environmental cleanup, the treatment method of introducing externally cultured bacteria to contaminated sites to clean up contaminated soil and groundwater is known as bio-augmentation. However, few cases have demonstrated the effectiveness of bioaugmentation to lead to complete purification. This is due to the reduction of the population size at the start time of augmentation in cases where bacteria released to the site cannot grow on pollutants. Previous studies have shown that bacterivorous protistan predation is the primary cause. Although there are many examples of studies on the degradability of purified bacteria themselves, there are few studies on the behavior of prey bacteria in predation, and there is much to be elucidated. In this study, we examined how the bacterial resistance to protistan predation is developed when bacterium coexists with a protist.

Previous studies have shown that bacteria added to groundwater and river water can reduce their number in very short periods, while bacteria inhabiting these environmental waters are not predatory to protist but persist. In other words, bacteria cultured in a laboratory and added for environmental cleanup are rapidly predated by indigenous bacterivorous protists, but indigenous bacteria are less susceptible to predation. Because indigenous bacteria originally coexist with indigenous protists, it is not surprising that they are resistant to protistan predation. Regarding the resistance of bacteria to predation, there have been reports such as a violacein producing bacterium and a bacterium with filamentous morphological change. These are observable changes in characteristics. On the other hand, there are no reports of general bacterial resistance to predation without such observable changes.
changes. Accordingly, we chose the phenol-oxidizing bacterium *Cupriavidus* sp. KN1\(^9\) as a model bacterium and investigated whether some predation resistance was developed when it coexisted with a protist. *Cupriavidus* sp. KN1 was used as the parent strain to make two transformants. They have two different drug resistances and fluorescent protein production capabilities, respectively. Thus the individual strains were distinguishably even when they were mixed. The two strains were then prepared under two different conditions, with and without predation, and then mixed to evaluate grazing-resistant traits.

2. EXPERIMENTAL METHODS

(1) Bacteria, protist, and plasmid used

Bacteria, a protist, and a plasmid used in this study are shown in Table 1. The protist used in predation experiments is the flagellate *Spumella* sp. TGKK2 (NBRC111014).\(^{10}\) The prey bacteria used are transformants of the wild-type strain *Cupriavidus* sp. KN1. *Cupriavidus* sp. KN1 is a phenol-oxidizing bacterium whose phenol hydroxylase has trichloroethylene degradability.\(^9\) The Tn5 transposon vector pUT\(^{11}\) was used to develop the transformants. Two transformants, *Cupriavidus* sp. KN1-TGF and *Cupriavidus* sp. KN1-KRF (preparation procedures described below), were constructed. *Cupriavidus* sp. KN1-TGF produces a green fluorescent protein and is tetracycline-resistant (Tc\(^r\)). *Cupriavidus* sp. KN1-KRF produces a red fluorescent protein and is kanamycin-resistant (Km\(^r\)). Thus, the two strains possess different drug resistances and fluorescent colors and can be selectively grown on an agar medium. They were measured by colony-forming unit (CFU) selectively and double-checked by their fluorescent color.

LB medium (Difco tryptone 10 g, Difco yeast extract 5 g, NaCl 5 g dissolved in 1 L of purified water, pH adjusted to 7.0) was used to cultivate strains derived from the strain KN1. Purified agar powder of 15 g/L was added to LB medium for colonization. Also, antibiotics were added to LB agar medium for the detection of the transformants.

For *Cupriavidus* sp. KN1-TGF, Tc was set at a final concentration of 2 \(\mu g/mL\) that was lower than an original value of 5g/mL, because the strain KN1-TGF was found to become less resistant to Tc in continuous experiments. For *Cupriavidus* sp. KN1-KRF, the final Km concentration was set at 300 \(\mu g/mL\) that was higher than an original value of 100 \(\mu g/mL\), because the strain KN1-TGF developed small colonies at that concentration. Plates were incubated at 30°C for 2 days.

(2) Introduction of fluorescent protein genes

The fluorescent protein genes used in this study were the green fluorescent protein genes ZsGreen in pZsGreen vector (Clontech Laboratories Inc.) and the red fluorescent protein gene tdTomato in ptdTomato vectors (Clontech Laboratories Inc.). As shown in Fig. 1, ZsGreen was paired with the Tc\(^r\) gene, and tdTomato was paired with the Km\(^r\) gene. Both were designed to be expressed from the *P1P2* promoter of the Tc\(^r\) gene in pBR322.\(^{12}\) Each of these genes was inserted into the multi cloning site of pUT, and the recombinant pUT transformed *Escherichia coli* SM10 Api.\(^{11}\) The transformant and *Cupriavidus* sp. KN1 were conjugated overnight on a membrane placed on LB agar medium at 30°C. The conjugated bacteria were diluted as appropriate and applied to an agar medium for the selection of a transformant. Thus the transformant *Cupriavidus* sp. KN1-TGF was selected. This agar medium is prepared by adding 2 mM sodium

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### Table 1 Bacteria, protist, and plasmid.

| Species or plasmid | Description | Reference or source |
|--------------------|-------------|---------------------|
| *Escherichia coli* | SM10 \(\lambda\)pir | 11) |
| *Cupriavidus* sp. | KN1 | 9) |
| | KN1-TGF | This study |
| | KN1-KRF | This study |
| *Spumella* sp. | TGKK2 | 10), NBRC111014 |
| Plasmid | pUT | 5.2kbp, Tn5 transposon vector, Ap\(^r\) | 11) |

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lactate and 5 μg/mL Tc to nutrient broth soytone yeast extract inorganic basal medium\textsuperscript{13} \cite{6} (hereinafter referred to as NSY-IB medium) that contains only inorganic compounds. Similarly, \textit{Cupriavidus} sp. KN1-KRF was obtained using the agar medium containing 100 μg/mL of Km instead of Tc.

(3) Preparation of prey bacteria

\textbf{a) Batch culture}

\textit{Cupriavidus} sp. KN1-TGF and KN1-KRF were cultivated in LB medium at 30°C with 200 rpm shaking overnight. The bacterial cultures were centrifuged (11,100 x g, 5 min, 4°C). The supernatants were discarded, and the cell pellets were suspended in NSY-IB medium. This procedure was repeated. Finally, the cells were resuspended in NSY-IB medium in a tube. Then it was shaken at 20°C and 200 rpm for a day.

\textbf{b) Continuous culture}

The continuous flow reactor was constructed by connecting two completely mixed reactors in series as shown in Fig. 2. The substrate supplied was NSY-IB medium containing 2 mM sodium lactate (=72 mg/L of TOC). The reactor’s working volume was 100 mL and the residence time was set at 30 hours (Q = 3.33 mL/hr). The temperature was set at 20 ± 1°C, and agitation in the tank was set at 400 rpm with a stirrer. The first reactor was a bacterial culture vessel in which sodium lactate in the medium was degraded and bacteria grow. The second reactor was a protistan culture vessel in which grown bacteria were predated by \textit{Spumella} sp. TGKK2. The effluent of the first reactor was collected from the sampling port 1, and the effluent of the second reactor was collected from the sampling port 2.

Lactate was measured by Shimadzu Prominence HIC-NS ion chromatography with Shodex IC NI-424 column. TOC was measured by Shimadzu TOC-V CHS analyzer. The protistan numbers were directly counted by microscopy for the effluent of the second reactor using a Toma’s hemocytometer. CFU and pH were also measured.

This continuous system operated two lines of Run1 and Run2 at the same time, using \textit{Cupriavidus} sp. KN1-TGF in Run1 and \textit{Cupriavidus} sp. KN1-KRF in Run2 as prey bacteria.

(4) Grazing-resistance evaluation

Experiments were performed on the evaluation of predation resistance using the aforementioned batch and continuously cultured bacteria. In the case of batch cultured bacteria, \textit{Cupriavidus} sp. KN1-TGF was preyed by \textit{Spumella} sp. TGKK2, and after 4 weeks, the protistan-uncontacted \textit{Cupriavidus} sp. KN1-KRF at the same level as the residual bacteria of KN1-TGF was added to assess which strain was more susceptible to predation. Similar experiments were performed by replacing bacterial strains. In the case of continuously cultured bacteria, the sample was collected for the predation experiment, assuming that the number of the residual bacteria and the number of the protist in the effluent of the second reactor reached a constant level at a steady state. The effluent from the second reactor of Run1 containing residual \textit{Cupriavidus} sp. KN1-TGF and \textit{Spumella} sp. TGKK2 was mixed with the effluent of the first reactor of Run2 containing \textit{Cupriavidus} sp. KN1-KRF not exposed to the flagellate to compare and evaluate the predation process. Conversely, the effluent from the first tank of Run1 was added to the effluent from the second tank of Run2, and the similar predation experiment was performed.

![Fig 2: Continuous flow reactors for bacterial and protistan culture.](image-url)
In predation experiments, a sterile polypropylene test tube of 15 mL volumes was used. The bacterial concentrations used ranged from $10^6$ to $10^9$ CFU/mL. Protistan concentrations were measured and adjusted with a Toma’s hemocytometer. The initial concentration of the flagellate TGK2 was adjusted to $10^3$ protist-cells/mL when bacteria from batch culture were used. When bacteria from continuous cultures were used, the flagellates in the effluent of the second reactor were used as-is. Predation experiments were performed on a shaking culture apparatus and shaken at 180 rpm, 20°C. The prey bacteria survived in a tube was also measured by CFU using LB agar medium containing antibiotics. *Cupriavidus* sp. KN1-TGF and KN1-KRF can be measured separately as described above.

3. **EXPERIMENTAL RESULTS**

(1) Evaluation of predation resistance using batch culture bacteria

As shown in Fig. 3A, *Cupriavidus* sp. KN1-TGF was adjusted to three different initial concentrations ($3 \times 10^6$, $3 \times 10^7$, $3 \times 10^8$ CFU/mL) and *Spumella* sp. TGK2 was added to a final concentration of $10^3$ protist-cells/mL. The results indicated that the residual bacterial count of KN1-TGF after 4 weeks was approximately $10^6$ CFU/mL, regardless of the initial bacterial concentration. On Day 28, the residual *Cupriavidus* sp. KN1-TGF was mixed with KN1-KRF that simultaneously prepared and stored at 4°C. Bacterial counts after 1 week showed that *Cupriavidus* sp. KN1-TGF remaining after predation had little change in bacterial counts, but the newly added *Cupriavidus* sp. KN1-KRF was reduced to initial levels of 2-3%.

In order to prove that this phenomenon was not due to the intrinsic properties of *Cupriavidus* sp. KN1-TGF and KN1-KRF, we reversed the combination of both strains and performed the same experiment. The equal level of *Cupriavidus* sp. KN1-TGF was added to *Cupriavidus* sp. KN1-KRF 4 weeks after the start. The results are shown in Fig. 3B. The reduction in each concentration of prey bacteria up to 4 weeks after the start of the experiment was approximately $10^6$ CFU/mL, similar to the previous experiment. Also, after 1 week of the addition of *Cupriavidus* sp. KN1-TGF, only the added bacteria showed a remarkable decrease, which was reduced to approximately 2% of the value at the time of addition.

The results of the experiments shown in Fig. 3 suggest that bacteria remaining after predation are less susceptible to predation than cultured bacteria that do not undergo predation. The survived bacterial cells seem to acquire some predation resistance during the 4 weeks with the protist.

(2) Evaluation of predation resistance using continuous culture bacteria

The results in the above-mentioned experiment suggest that some predation resistance is conferred on residual bacteria after the predation. Two series of the continuous flow experimental apparatus shown in Fig. 2 were operated to conduct another grazing-experiment. Table 2 shows the mean and standard deviation of the four measurements of water quality at 20-29 days, assuming a steady-state of 9 days. Run1 and Run2 resulted in almost the same water quality. The pH was approximately 8, and when the vessel proceeded from the first tank to the sec-

![Fig.3](image-url) Analysis of grazing-resistant of batch cultivated bacteria with and without a history of protistan predation.
ond tank, it decreased slightly. Sodium lactate was almost consumed in the first reactor, and ion chromatography showed a detection limit of 0.01 mM or less. Therefore, the concentration of organic matter in the reactor was monitored with TOC. TOC remained at around 6 mg/L in the first reactor but increased to around 11 mg/L in the second reactor. It is likely that some soluble TOC components were eluted in the solution by predation. The consumption of 2 mM lactic acid increased the bacterial count in the first reactor to about $2 \times 10^8$ CFU/mL, whereas in the second reactor, protistan predation reduced the bacterial count to 2% of the value in the first reactor. Resultantly protistan number increased to approximately $8 \times 10^5$ protist-cells/mL with conversion rates around 230 CFU/protist-cell. Thus, in the period 20–29 days, assuming steady-state, each measurement showed similar values in two series of Runs.

On Day 26 (20.8 times HRT) after the start of the operation, samples from the sampling port 1 and 2 shown in Fig. 2 were collected. Predation experiments were performed by mixing effluents from the second and first reactors of Run1 and Run2, respectively. In the experiment shown in Fig. 4A, the first reactor effluent of Run2 was added to the second reactor effluent of Run1. On the other hand, in the experiment shown in Fig. 4B, reversely, the first reactor effluent of Run1 was added to the second reactor effluent of Run2. The measurements in each experiment were carried out in duplicate.

In Fig. 4A, *Cupriavidus* sp. KN1-KRF derived from the effluent of the Run2 first reactor was rapidly predated, and after 1 week, *Cupriavidus* sp. KN1-KRF was reduced to 0.1-0.2% of the initial value. On the other hand, the decrease in *Cupriavidus* sp. KN1-TGF was slow. It coexisted with the protist in the second reactor of Run1. After 1 week, the bacterial number decreased to 25-50% of the initial value. Residual values were also about 100 times greater for *Cupriavidus* sp. KN1-TGF with a predatory history. Thereafter, the residual values of *Cupriavidus* sp. KN1-TGF decreased slowly, however, that of *Cupriavidus* sp. KN1-KRF remained almost constant at a low level. Fig. 4B is the result of an inverse experiment with the combination of *Cupriavidus* sp. KN1-TGF and KN1-KRF. *Cupriavidus* sp. KN1-TGF that had no predation history was grazed at a very fast rate compared to *Cupriavidus* sp. KN1-KRF with a predation history. The residual rate of *Cupriavidus* sp. KN1-KRF exposed to the protist after 1 week was reduced to 14-18% which was smaller than the results in Fig. 4A. However, other trends were quite similar. Thus, it became clear that, even in the same bacterial species, those with the history of the protistan predation had acquired some grazing-resistant traits.

Table 2 Water quality of continuous flow reactor effluents under presumable steadystate.

|          | pH | *TOC (mg/L) | CFU/mL | Protist (cells/mL) | CFU/protist-cell | Conversion rate |
|----------|----|-------------|--------|-------------------|-----------------|-----------------|
|          | 1st Reactor Eff | 2nd Reactor Eff | 1st Reactor Eff | 2nd Reactor Eff | 1st Reactor Eff | 2nd Reactor Eff | 1st Reactor Eff | 2nd Reactor Eff | 1st Reactor Eff | 2nd Reactor Eff |
| Run1     | 8.26±0.12 | 8.15±0.02 | 6.74±0.39 | 10.97±0.86 | 1.77±0.04E+08 | 3.84±1.02E+06 | 7.86±1.14E+05 | 224±37 |
| Run2     | 8.28±0.08 | 8.16±0.03 | 5.60±0.23 | 11.14±1.27 | 1.99±0.06E+08 | 5.42±2.83E+06 | 8.46±1.01E+05 | 231±27 |

* TOC: supernatant of a sample was used after centrifuge (11,100 x g, 10 min, 4°C) as soluble TOC.

Fig.4 Analysis of grazing-resistant of continuous cultivated bacteria with and without the history of the protistan predation.
(3) Comparison of bacterial decrease in predation experiments

Two types of grazing experiments, using batch and continuously cultured prey bacteria, were shown in Fig. 3 and Fig. 4. The apparent decrease rate constant calculated from these data was obtained to compare all results. The rate of reduction of the predated bacterium by one week after the addition of the bacteria not exposed to the flagellate was assumed to follow the first-order reaction. In Fig. 3, the apparent decrease rate constant from Day 28 to Day 35 was calculated, and in Fig. 4, the apparent decrease rate constant from Day 0 to Day 7 was calculated. In Fig. 3, for bacteria without a history of predation, the mean of six apparent decrease rate constant k (day\(^{-1}\)) was calculated for a total of six data sets, three sets in A (closed symbols), and three sets in B (open symbols). The values of k were calculated and averaged for the remaining six data sets with a history of predation in the same manner. Finally, the results of the bar graph on the left in Fig. 5 were obtained. The bacteria without a history of predation were predated about nine times faster than those with a history of predation. In Fig. 4, a similar analysis was performed on four bacterial decreases without a history of predation, and four bacterial decreases with a history of predation. The mean values can be seen from the bar graphs to the right of Fig. 5. The bacteria without a history of predation decreased approximately four times faster than those with a history of predation. The final bacterial counts were also summarized in Fig. 6. The numbers of final residual bacteria were different in experiments with batch culture bacteria and experiments with continuous culture bacteria. The numbers of residual bacteria in the former case were 10 times higher than those in the latter case. In the same tube, however, bacteria with a history of predation remained at concentrations approximately 10 times higher than those without a history of predation.

Fig. 6: Final concentrations of bacteria with and without predation history.

4. DISCUSSION

Regarding bacterial predation in protist, there is a review of changes in predation resistance, mainly with changes in shape and size.\(^{14}\) As a specific example, it has been reported that, in the presence of a protist, Flectobacillus sp. GC-5 varies its shape into a long filament, increasing its predation resistance.\(^{8}\) On the other hand, there are no evaluation methods and no reports on the formation of predation resistance in the absence of morphological changes.

In this study, Cupriavidus sp. KN1 was used to evaluate the development of grazing-resistant traits to the protist. Regarding this bacterium, it was not possible to observe morphological changes due to contact with the protist. Therefore two distinctly detectable transformants were developed from this bacterial strain. The two transformants were cultivated as prey bacteria under conditions with the presence or absence of the protist and then mixed to assess their resistance to the protistan predation.

In the first experiments, the batch cultivated Cupriavidus sp. KN1-TGF and KN1-KRF ranging from 3 × 10^6 to 3 × 10^6 CFU/mL were grazed by Spumella sp. TGKK2 as shown in Fig. 3. In this experiment, the number of residual bacteria after 4 weeks remained at...
a constant level of approximately $10^6$ CFU/mL, regardless of the initial bacterial count. The results of this experiment suggest that there may be a constant lower limit for the reduction due to predation. Next, a second strain with no history of contact with the protist was added to each of the remaining bacteria on Day 28 so that the concentration was at the same level. Then the reduction process was compared and evaluated. The results suggested that the second strain without the history of the protistan contact was preferentially predated showing that the first strain had been exposed to the protist was conferred some predation resistance. The final concentration reached on Day 49 was also about 10-fold higher in strains with a history of contact and about $10^9$ CFU/mL. Morphological changes of the pray bacteria have not been observed during the experiments.

In order to verify this phenomenon, an additional experiment was conducted. Two completely mixed reactors were connected in series. A comparison of prey properties was performed by using a bacterium from the first reactor without the protist and a bacterium from the second reactor containing the protist. The results were shown in Fig. 4. It was found that bacteria in the first reactor, which were not in contact with the protist, were more susceptible to selective predation, and that residual bacteria with the history of the protistan contact in the second reactor were more resistant to the predation.

Thus, both batch and continuous cultures of bacteria with the history of the protistan contact showed a slower reduction and a higher final residual concentration, indicating some predation resistance in bacteria. To compare the rates of reduction, the mean values of the apparent decrease rate constants were calculated for each of the batch and continuous culture systems for the one-week reduction immediately after the mixing of bacteria with and without the protistan contact. The apparent decrease rate constants for all tests were summarized in Fig. 5. The experiments using the batch culture show that the absolute value of the apparent decrease rate constant $k$ for bacteria with a history of predation is approximately 10 times lower than that for bacteria without predation. In the same manner, in the experiments using the continuous culture, the absolute $k$ value was reduced to approximately one-fifth with a history of predation.

The final residual bacterial concentration in all studies was also summarized as shown in Fig. 6. From this figure, a clear difference was observed in the number of final residual bacteria between bacteria with and without the history of the protistan contact. The final total bacterial count in the experiment using continuous culture was approximately $10^5$ CFU/mL. This value is almost 10 times lower than that in the experiment using the batch culture. This is probably due to the about 800-fold increase in protistan concentrations at the start of this experiment at approximately $8 \times 10^5$ protist-cells/mL compared to $10^5$ protist-cells/mL in the experiment using the batch culture. Anyhow, the concentration of residual bacteria was increased by contact with the protist in both experiments, suggesting that the protistan contact confers some grazing-resistances.

After the predation, remained bacteria were then isolated, cultured, and re-ingested by the protist. They were predated as quickly as bacteria without a history of predation (data is not shown). Thus, the observed resistance to predation was considered to be changes in cell conditions rather than the appearance of mutants. Further elucidation of the detailed causes of these changes in the conditions and the mechanisms involved in which genes are connected is an issue to be addressed in the future.

In the past, we conducted an external culture of *Cupriavidus necator* KT-1 for purification of trichloroethylene-contaminated soils and injected it into contaminated groundwater. At this time, it was confirmed that the injected bacteria decreased significantly faster than other indigenous bacteria and that the microbial community structure of indigenous bacteria recovered rapidly. Furthermore, in a different experiment, when *Cupriavidus necator* KT-1 was added to river water, it was found to be predated more quickly than indigenous bacteria in river water. Therefore, it is possible to explain these results if indigenous bacteria in the natural environment, which are constantly in contact with protists, already possess resistance to protistan predation.

In order to prove this hypothesis, it is necessary to investigate the broadness of predation resistance phenomena by increasing the types of bacteria derived from the natural environment to be evaluated. In addition, by examining not only bacteria derived from the natural environment but also bacteria derived from a clinical origin, such as *Escherichia coli*, it is also an interesting question whether this phenomenon is a common phenomenon that can be caused by a wide range of bacteria, regardless of their origin.

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