Master's Thesis

Cyanide, An Environmental Inhibitor of Predation by *Bdellovibrio bacteriovorus* HD100

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Abstract

BALOs are predatory bacteria attacking specific Gram-negative prey bacteria and have been studied about both their life itself and their possibility to be used as alternatives to antibiotics. One of most widely used strain is a Bdellovibirio bacteriovorus HD100. In this study, environmental factors affecting on competition between prey and predatory bacteria, cyanide, is studied. The results show cyanide can inhibit predation by BALO and how the cyanide effect on predation.

Contents

Chapter 1. Interaction and Competition between Bacteria: Quorum sensing, Antibiotics, and Predation

1.1 Summary

In bacterial world, many of interaction and competition have been occurred since long time ago, for getting a limited amount of resources and spaces for their own species. By recognizing same species, competitors, and co-existers with some of the cell-to-cell communication methods (e.g. quorum sensing), bacteria can perform their own interacting methods: giving antimicrobial agents outside, emission of wastes of metabolic pathway, and even prey upon other bacteria. In this chapter, some of these interactions of bacteria with each other would be explained, including related studies and papers.

1.2 Quorum sensing and Secondary metabolites

1.2.1 Quorum Sensing

Bacteria, organisms that individually reproduced by binary fission majorly, have been competing each other and their neighbors with resources and nutrients. Interacting and competition of bacteria accompany with “recognition of others” of course, and many behaviors needing more than one individual cell, for example, conjugation, symbiosis, secondary metabolism exist and were found. Such that the way of recognition was assumed, for instance, small and diffusible chemical substances for cell communication between bacteria [1].

Firstly, it was called as pheromones, the meaning of transferring and excited molecule [2]. Nevertheless, nowadays the term of ‘autoinducer’ is more widely used, due to their function of stimulating themselves to promote the production of pheromone. Autoinducers are used to do signaling each other and if it overcomes specific concentration, meaning that bacteria reach at considerable population, following behaviors get started, e.g. production of secondary metabolites. To say it simply, sensing pheromones are used to check environmental factors whether and how their own and/or other species exist, and bacteria change their actions as responses.

This kind of phenomena was named as ‘quorum sensing’ [3]. From and with this paper, quorum sensing has been in the spotlight of the research field. This kind of co-beneficial behavior for own species and characteristic that performed only if a large quantity of cell number is satisfied are somehow viewed as similar to multicellular organisms. Additionally, it is also studied with intra- and interspecies communication [4].

1.2.2 Antibiotics

One of the secondary metabolites produced and regulated by quorum sensing is antibiotics, for example, in *Actinobacteria* [5] and *Erwinia* [6], including antifungal substances such as

polyketides [7].

Some signaling molecule like γ-butyrolactones(GBLs) are also regulating production and differentiation of antibiotics in *Streptomyces* [8, 9]. To be specific, IM-2 (one of GBL family) controls nucleoside antibiotics manufacturing in *S. lavendulae* [10], and streptomycin is regulated by A-factor in GBL likewise in *S. griseus*. Similarly, signaling molecules named virginiae butanolides to induce making virginiamycin in *S. virginiae* [11]. Another signaling molecule, methylenomycin furans (MMFs) is known to be related with quorum sensing and coordinate methylenomycin assembly [12].

As stated above, there are many cases of antibiotic production controlled by quorum sensing and signaling molecules.

1.2.3 Cyanide

Cyanide is a chemical substance can be used as a strong toxin, which can kill an adult human with several mg of cyanide, with inhibition of cytochrome c oxidase majorly [13]. This substance can affect for animals having circulatory and nervous system such as insects, mice [14], and also *D. melanogaster* [13], with the amount of virulent *P. aeruginosa* producing. Actually, cyanide concentration manufactured by *P. aeruginosa* can be fatal for nematode, *C. elegans* [15, 16].

It is natural that cyanide is one of the secondary metabolites, and have related characteristics. It is related quorum-sensing as well. For example, in *Pseudomonas aeruginosa*, hcnABC is controlled transcriptionally by quorum sensing regulators, LasR and RhlR [17]. The cyanogenic bacteria, in this case, proteobacteria, have resistance to cyanide and the production is thought to have ecological functions to give advantages for producer [18]. As one of the examples, *P. flurescens* CHA0 give protection of root disease to nearby plants caused by fungi, and this function is related to hydrogen cyanide production [19]. Some of *Pseudomonas* strains, for instance, *P. fluorescens and P. putida.* produce cyanide for somehow plant-beneficial ecologically, for example, killing larvae [20]. In addition, cyanide production is also regarded as mobilization of metal ions with cyanide complexes by cyanogenic bacteria [21].

With this kind of high toxicity of cyanide for many animals, however, it is well-produced by *Pseudomonas* and some of *Chromobacterium* species. The precursor of cyanide in oxidative decarboxylation is considered as majorly glycine, in addition to glutamate and methionine as well [22, 23]. It is regarded as produced in early stationary phase [24]. HCN synthase is based on amino acid dehydrogenase/oxidase by sequence [25], and the major two mechanisms proposed as bacterial cyanogenic reactions: Firstly, glycine changed into hydrogen cyanide and carbon dioxide, with imino acetic acid as intermediate molecule. It is depend on HCN synthase (HcnABC) [26]. Secondly, L-amino acid oxidase and peroxidase makes L-histidine and oxygen molecules into imidazole aldehyde [27]. The main mechanism to make cyanide in proteobacteria is regarded as first one, with HCN

synthase. This enzyme is bound in cell membrane flavoprotein [28].

The metabolism of *Bdellovibrio* has been studied, with its distinct characteristic to grow inside intraperiplasmic space of prey, and the fact that *Bdellovibrio* is an obligate aerobe, being necessary for both prey-dependent growth [29] and prey-independent growth of host independent case [30] to get oxygen. *Bdellovibrio* strains have cytochrome a and c component [30] which are related to oxygen-consuming metabolisms, and it was studied that amino acid and phosphate uptake, as energy-dependent processes by *Bdellovibrio* can be inhibited by cyanide and carbonyl cyanide p-trifluoromethoxyphenyl hydrazine [31]. ATP uptake decrease [32] and amino acid uptake decrease [29] of *B. Bacteriovorus* 109J by cyanide is observed as well. Cyanide is treated as one of the inhibitory effectors (e.g. cyanide, azide, or arsenate) for energy-producing mechanisms of *Bdellovibrio* [33]. Therefore, it is obvious that cyanide would be negative effects for life of *Bdellovibrio* also.

1.2.4 Predatory bacteria

Bdellovibrio bacteriovorus is one of the predatory bacteria which is capable of attacking a broad range of Gram-negative prey bacteria, such as some of *Escherichia coli*, *Acinetobacter baumannii, Kelbsiella pneumoniae,* and *Yersinia* strains.

They and related predatory bacteria are called as *Bdellovibrio*-and-like-organisms(BALOs), and BALOs have been studied about both their life itself and their possibility to be used as alternatives to antibiotics. One of the most studied and widely used BALO species *is Bdellovibrio bacteriovorus* HD100.

All organisms in nature have been interacting and fighting each other to survive, with constant variation and development of their tools. In the world of microorganisms, they also interact and attack each other. For examples, they recognize others by quorum sensing and attack others by toxic compounds including antibiotics, and even predation like carnivores. These kinds of competition in nature can be utilized by humans, for dealing with worldwide rampancy of multidrug-resistant bacteria.

BALOs have various hydrolytic enzymes to break down inside composition of host bacteria, and utilize its nutrients to be elongated and divided to make new cells, while other predation forms exist: epibiotic predation by *V. chlorellavorus* [34], *M. aeruginosavorus* and *B. exovorus* [35]. BALOs have distinct and regular predatory cycle: first is attack phase that BALOs move to find host cells and intraperiplasmic growth phase that BALOs grow after invasion inside hose cell. With enough growth time, BALO cells would present septation and lysed out from host cells to find another victim [36, 37].

Predation can be achieved by one cell or by cell group. Individual predatory cells may eat prey in an epibiotic way or in invading way. In *Bdellovibrio* case, the endobiotic(invading) way is the method. Some predators (e.g. *Lysobacter spp., Myxobacteria)* could hunt by the group, with the

ability to do that in the single cell also.

Because of BALOs' ability to decrease pathogen and control population of bacteria with or without antibiotic resistance, it has been assumed as one of the candidates for alternatives to antibiotics, and it is also can be used in other fields like experiments about ecology and evolution [38].

Chapter 2. Predation study of *B. bacteriovorus* HD100 with *Chromobacterium piscinae* - Cyanide Negatively effects on Predation

2.1 Summary

Chromobacterium piscinae, one of *Chromobacterium* strain, produces violacein and cyanide. We tested these *Chromobacterium piscinae* for predation test by *Bdellovibrio bacteriovorus* HD100, and it showed delayed or blocked predation of HD100. We investigated what is precisely negative factors for BALO predation produced by *Chromobacterium piscinae*, such that we tested supernatant of *Chromobacterium piscinae* for predation test by HD100, with *Escherichia coli* MG1655 as prey.

The result suggested supernatant of *Chromobacterium piscinae* has predation inhibitory factor(s). We listed possible chemical substances as candidates and found that cyanide molecule can inhibit BALO predation, from mild inhibition (50μM) to complete blocking (400μM, until 96hr predation). It is double-checked by examining the cyanide-producing amount of *Chromobacterium piscinae* in nutrient media, until 24hr. Consequently, we can know that cyanide is one of the crucial factors to hinder *Bdellovibrio bacteriovorus* HD100 predation.

2.2 Introduction

BALOs are predatory bacteria eating on certain Gram-negative bacteria, and their habitats and preys are variable. They can be found in soil, plant roots, seawater, freshwater, biofilms, and sewage [39, 40], including in the animal gut [41]. So BALOs can predate much preys they can, however, there are also effectors to inhibit predation, such as indole [42], high concentrations of glucose or glycerol, low pH [43], and cyanide.

At the start of this chapter, it was clear that *C. piscinae* showed delayed and blocked predation by *B. bacteriovorus* HD100 when given a few nutrients by dilute nutrient broth but was predated when cultured in HEPES buffer.

2.3 Materials and Methods

2.3.1 Delayed Predation of HD100 with *C. piscinae*

Four independent *C. piscinae* and four independent *B. bacteriovorus* HD100 were cultured overnight. HD100 was used after soft agar plating inoculation and liquid cultured two times before overnight culture, based on revised culturing method [44], changed to use HEPES (purchased from Sigma Aldrich) buffer amended with MgCl2 and CaCl2 for liquid culture.

After culturing *C. piscinae* in NB (Nutrient broth, Neogen) for 24hr with shaking (250rpm, 30'C), the culture was centrifuged (15min, 1878 rcf) to be pelleted and resuspended with HEPES buffer until OD600 reaches at 1.0. Then it was divided into two tubes (Tube A and Tube B) and centrifuged again. Tube A was resuspended with HEPES, and tube B was resuspended with DNB(Diluted Nutrient Broth;10-fold diluted nutrient broth) both for adjusting OD600 to 1.0. Both tubes were divided into two tubes including 10ml of resuspended culture each, as a BALO tube and a control tube.

Initial CFU and PFU were checked, and 0.45μm filtered *B. bacteriovorus* HD100 100ul was added to each tube of 10ml *C. piscinae* suspension for predation test. CFU of *C. piscinae* and PFU of *B. bacteriovorus* HD100 of tubes were checked at 24hr and 48hr after predation. It was the key point that filtering culture with 0.45μm before PFU to remove the C. piscinae cells which block plaque forming.

2.3.2 *C. piscinae* supernatant have negative impact on HD100 predation

B. bacteriovorus HD100 predation of *E. coli* MG1655 was tested, resupended with supernatant of *C. piscinae* cultured in DNB or HEPES. Same liquid culturing method above was used for *B. bacteriovorus* HD100 after soft agar plating inoculation and liquid cultured two times before overnight culture. *E. coli* MG1655 was cultured with LB broth (Difco™ LB Broth, Miller (Luria-Bertani)), shaking incubated overnight at 37'C, 250rpm. *C. piscinae* was overnight cultured in NB with shaking condition (30'C, 250rpm), and 1:100 diluted inoculation was done in HEPES or DNB

media before overnight culturing (30'C, 250rpm). Both *E. coli* and *C. piscinae* were centrifuged (15min, 1878 rcf) to get a pellet. Supernatant of *C. piscinae* was collected with 0.22μm filtering. *E. coli* MG1655 was resuspended with filtered *C. piscinae* supernatant (from HEPES or DNB culture) until OD600 reached at 1.0. Resuspended *E. coli* MG1655 was divided for 10ml each into 50ml tube (Falcon), and 100ul of overnight cultured *B. bacteriovorus* HD100 was inoculated. All tubes were shaking incubated at 30'C, 250rpm. CFU was checked at initial point(0hr) and 24hr.

Preparation of dose-dependent treatment of *C. piscinae* supernatant was similar as above, nevertheless, one group of overnight cultured *E. coli* MG1655 was resuspended with HEPES buffer up to 2.0 of OD600 value, then mixed with the same volume of *C. piscinae* DNB supernatant. It was 50% supernatant group and serially diluted into 25% and 12.5% also. Another group of cultured *E.coli* MG1655 was resuspended only with *C. piscinae* DNB supernatant. It was 100% supernatant group. Last group, 0%(HEPES) group of *E. coli* MG1655 was only resuspended with HEPES buffer. Resuspended *E.coli* MG1655 was divided for 10ml each into 50ml tube (Falcon), and 100ul of overnight cultured *B. bacteriovorus* HD100 was inoculated. All tubes were shaking incubated at 30'C, 250rpm. CFU was checked at initial point(0hr) and 24hr.

2.3.3 Violacein Does Not Inhibits HD100 predation

Extract violacein was extracted by ethanol from *C. piscinae* and purified by acetone crystallization method, similar in [45], nonetheless, with an evaporator in this experiment. 0.05g/L and 0.5g/L violacein stock (100X) in DMSO were prepared. Overnight cultured *E. coli* MG1655 was resuspended in HEPES to adjust OD600 value to 1.1. Violacein stock was added as 1:100 ratio for meet each concentration (0.5mg/L and 5mg/L). Overnight cultured *B. bacteriovorus* HD100 (liquid cultured at least twice before experiment) was inoculated as 1:100 ratio. All tubes were shaking incubated at 30'C, 250rpm. CFU and PFU were checked at initial point(0hr) and 24hr.

2.3.4 Purged Supernatant of *C. piscinae* Losing Inhibitory effect for HD100 Predation

C. piscinae was overnight cultured in NB with shaking condition (30'C, 250rpm), and 1:100 diluted inoculation in DNB was done to culture overnight (30'C, 250rpm). Centrifugation (15min, 1878 rcf) and 0.22μm filtering were done to get supernatant without cells. These supernatants were poured 20ml each into 60ml serum bottles and purged with nitrogen gas (flow rate: 3L/min) for 1hr. Then overnight cultured *E. coli* MG1655 was resuspended with purged supernatant, followed by 1:100 ratio inoculation of overnight cultured *B. bacteriovorus* HD100 (liquid cultured at least twice before experiment). CFU was checked at initial point(0hr) and 24hr.

2.3.5 Measurement of Cyanide concentration

Modified method from [46] was used, based on a paper [47]. 0.1M o-dinitrobenzene (Fluka) and 0.2M p-nitrobenzaldehyde solutions in 2-methoxyethanol were prepared. Solvent of two solutions was 2-methoxyethanol, and these substances were purchased from Sigma Aldrich. For each measurement, a fresh 1:1 mixture of 0.1M o-dinitrobenzene (Fluka) and 0.2M p-nitrobenzaldehyde was made. Then, 23µl portions of the diluted measuring samples were located in 1.5ml microcentrifuge tubes, and 77µl of the mixture was added, followed by incubation for 30 min at room temperature. Next, 900µl of 2-methoxyethanol was added to each tube, and the well-mixed 100µl liquid inside 1.5ml tubes was put in wells of 96-well transparent plate. Optical density was measured at 578 nm for each well including samples. Concentrations were obtained by comparison with a calibration curve computed using serial dilutions of a KCN stock solution.

2.3.6 Different concentration of Potassium Cyanide Inhibits HD100 predation

From 1M KCN solution made in sterile HEPES, serially diluted concentration (1M -> 100mM -> 10mM -> 1.6mM -> 800µM -> ... -> 100µM) of KCN were prepared to use as 2X solutions in this experiment. Prey, overnight cultured *E. coli* MG1655 was prepared as 2.0 of OD600 value in HEPES. Each of prepared 2X KCN solution and adjusted *E. coli* MG1655 were mixed as 1:1 ratio and final volume was 10ml each in a 50ml tube (Falcon). 1:100 ratio inoculation of overnight cultured *B. bacteriovorus* HD100 (liquid cultured at least twice before experiment) for each tube was followed. All tubes were shaking incubated at 30’C, 250 rpm. CFU and PFU of tubes were checked at initial point(0hr) and 24hr.

2.4 Results

2.4.1 Delayed Predation of HD100 with *C. piscinae*

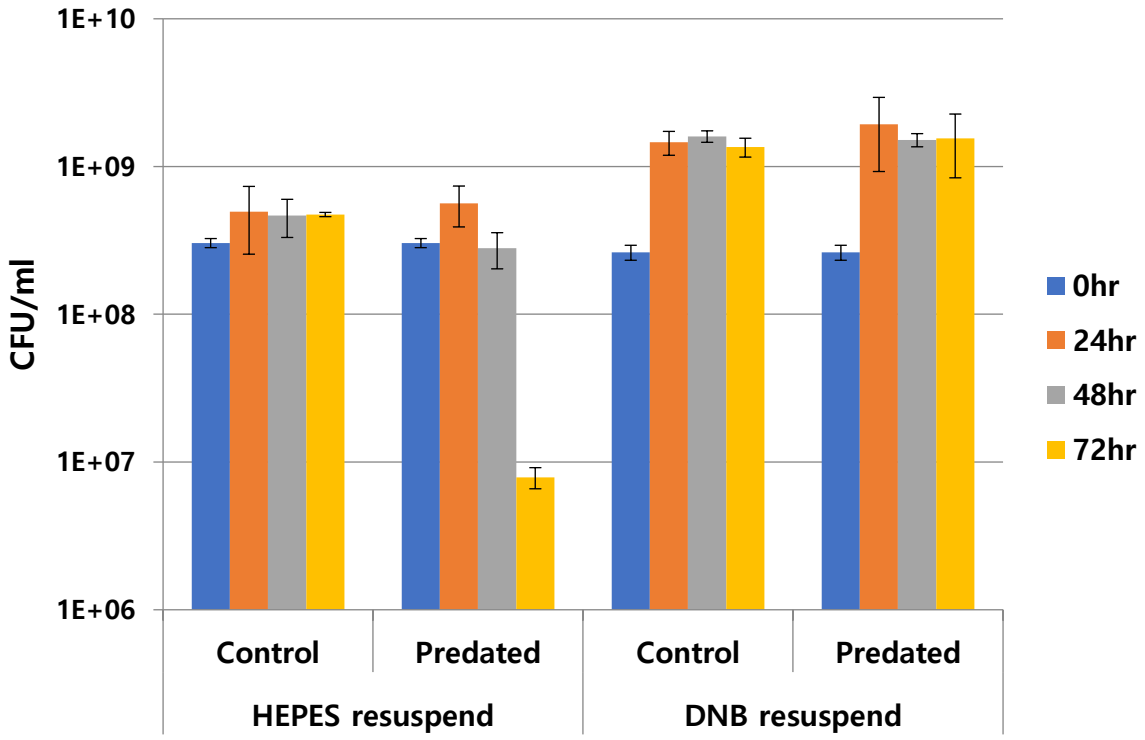


Figure 2.1. Inhibited Predation of *B. bacteriovorus* HD100 with *C. piscinae* in HEPES and DNB media. Left and right side of the graph above divided as resuspending media of *C. piscinae.* Control groups were not included predator (*B. bacteriovorus* HD100), and predated groups contained active *B. bacteriovorus* HD100.

Unlike the well-predated prey, *E. coli* MG1655, *C. piscinae* showed slower predation. It showed slower predation and predated after 72hr incubation when it was resuspended with HEPES, and not predated until 72hr when it was resuspended with DNB.

2.4.2 *C.piscinae* supernatant have negative impact on HD100 predation

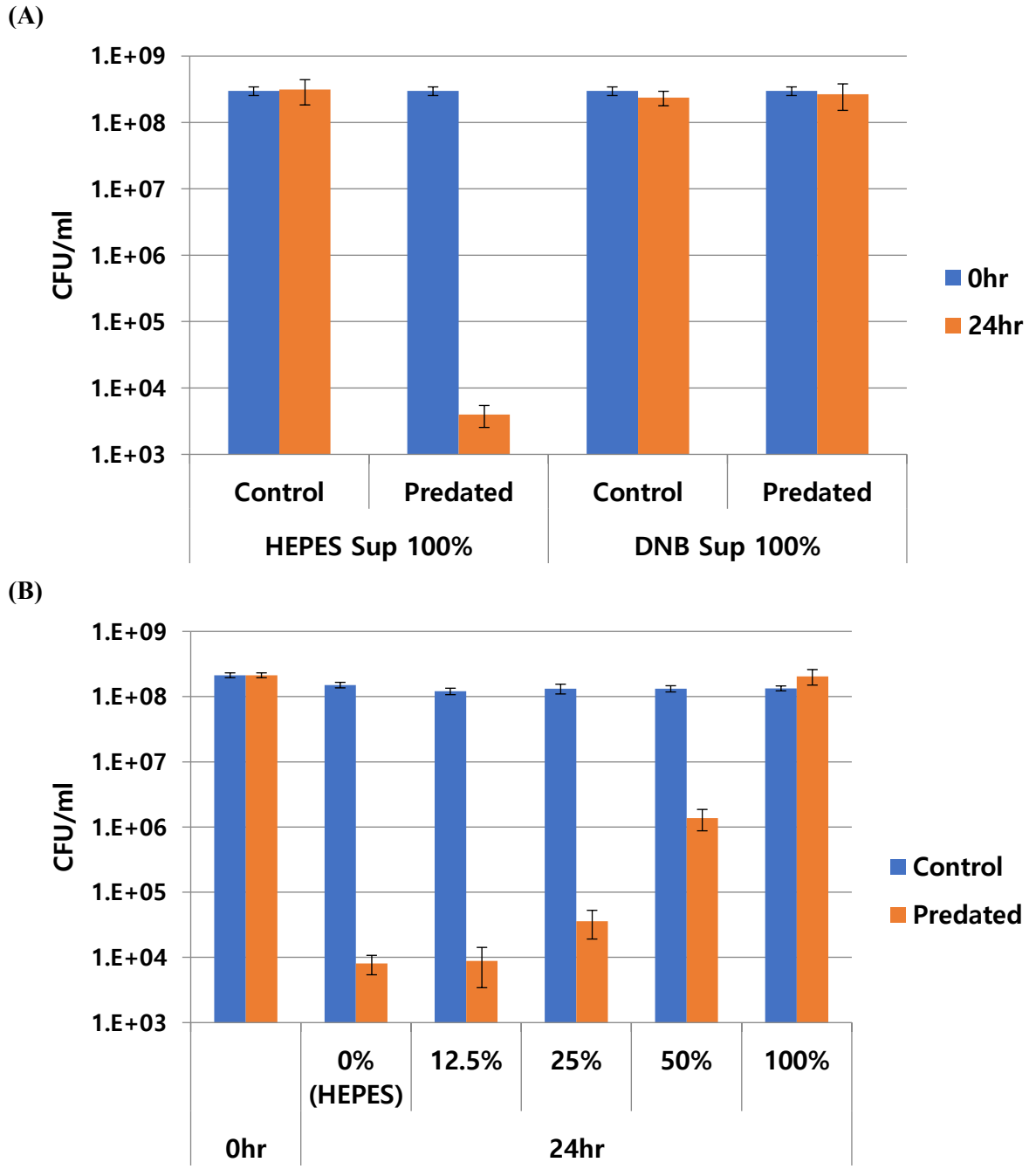


Figure 2.2. *C. piscinae* supernatant have negative impact on B. bacteriovorus HD100 predation. (A) *E. coli* predation in *C. piscinae* media from HEPES and DNB. *E. coli* MG1655 was predated by *B. bacteriovorus* HD100, with *C. piscinae* supernatants (cultured from HEPES or DNB) as a resuspending media. Control groups did not put HD100, and predated group was added HD100. (B) Dose-dependent inhibition of *E. coli* predation by *C. piscinae* media (DNB). Overnight cultured supernatant of *C. piscinae* from DNB media was used, and serial diluted to see inhibitory effect for *B. bacteriovorus* HD100 predation. Dilution was done with HEPES buffer.

E. coli MG1655 cells were very slowly predated in (A) of Fig 2.2, even not predated until 72hr in a group of resuspended *C. piscinae* DNB supernatant. It was clear that DNB supernatant of *C. piscinae* have inhibitory effect, though we did not know what exactly it was. It was also proved with the dose-dependent test in (B) of Fig 2.2. Such that, further study to investigate inhibitor proceeded.

2.4.3 Violacein Does Not Inhibits HD100 predation

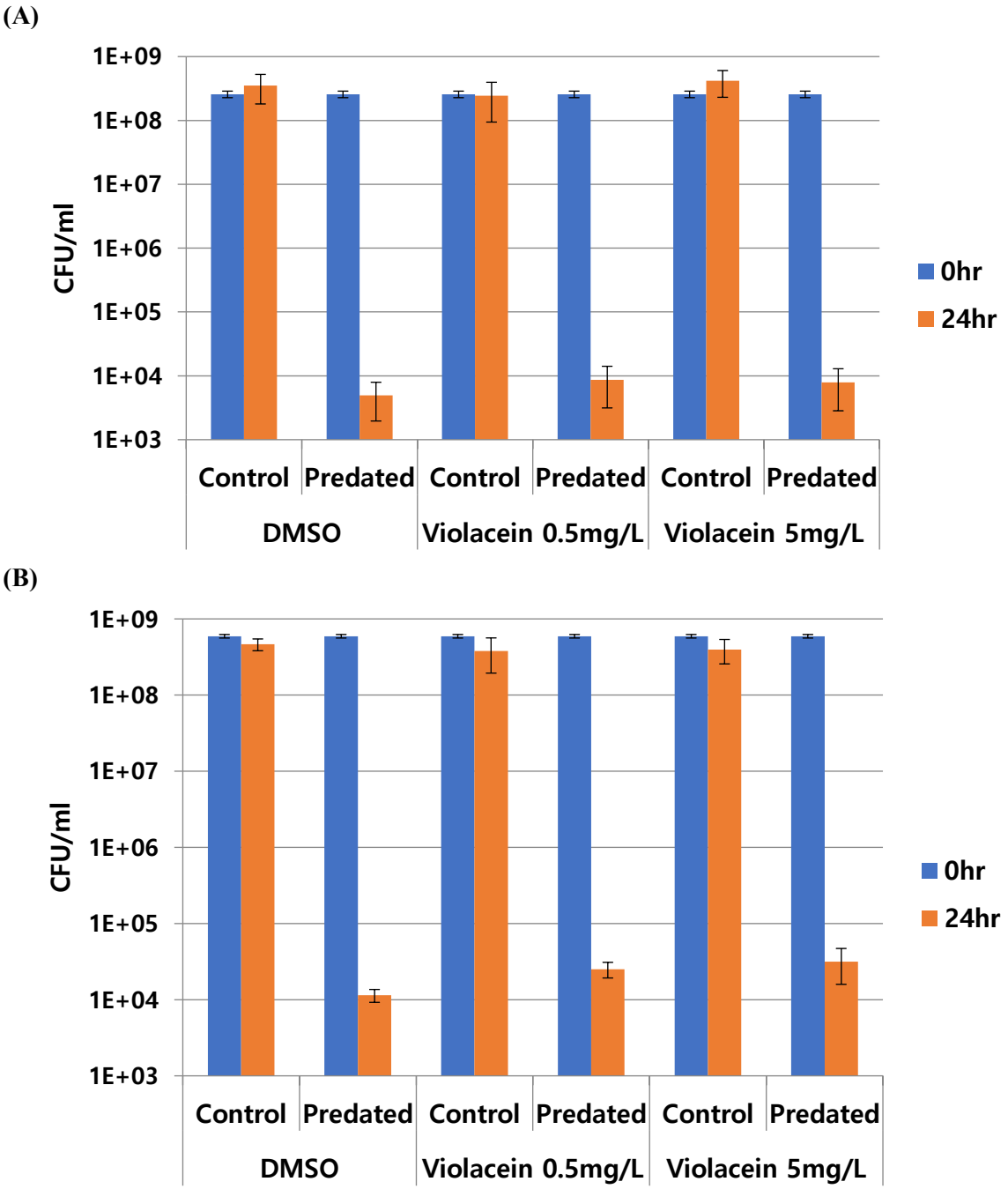
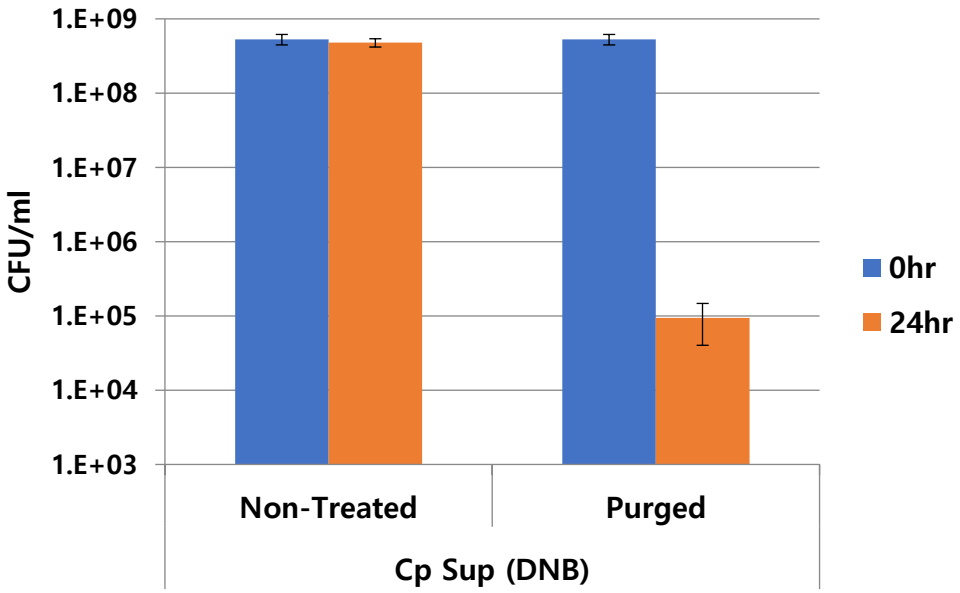


Figure 2.3. Violacein does not inhibits *B. bacteriovorus* HD100 predation. Control group did not contain overnight cultured *B. bacteriovorus* HD100, and predated group was added HD100. (A) *E. coli* MG1655 predation with crude extracted violacein. (B) *E. coli* MG1655 predation with pure violacein.

As shown in Fig 2.3, both extracted violacein from *C. piscinae* and purchase violacein (Sigma Aldrich) indicated no inhibitory effect at all toward *B. bacteriovorus* HD100. Such that, further investigation proceeded.

2.4.4 Purged Supernatant of *C. piscinae* Losing Inhibitory effect for HD100 Predation

(A)



(B)

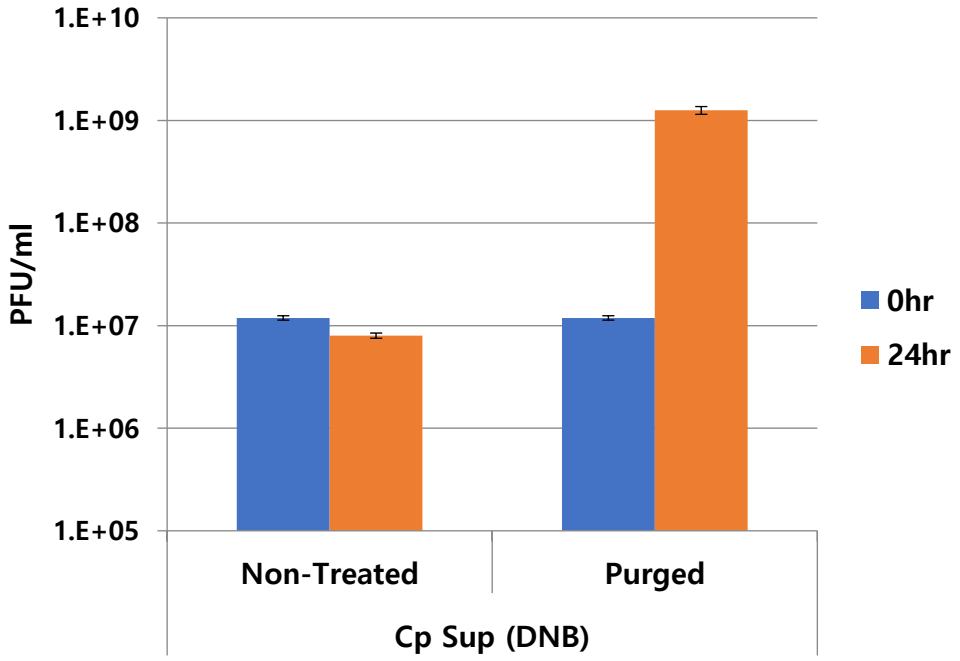


Figure 2.4. Purged supernatant of *C. piscinae* losing inhibitory effect for *B. bacteriovorus* HD100 Predation. (A) is CFU, and (B) is PFU graph for *E. coli* MG1655 predation by *B. bacteriovorus* HD100, resuspended with *C. piscinae* supernatant from DNB media, with or without purging. Left(Non-treated) groups show *E. coli* MG1655 with *C. piscinae* DNB media with *B. bacteriovorus* HD100, and right(Purged) groups indicate similar with non-treated group except resuspending media was changed as purged *C. piscinae* DNB media.

Figure 2.4 explains about how purging was affecting on the inhibitory effect of *C. piscinae* supernatant from DNB media. It was obvious that major inhibitor in *C. piscinae* supernatant was disturbed by 1hr purging by nitrogen gas. Therefore, we could guess that inhibitor of the supernatant is volatile. Such that, the volatile substance was presumed and predicted as an inhibitor in the continued experiment.

2.4.5 Cyanide Production of *Chromobacterium piscinae*

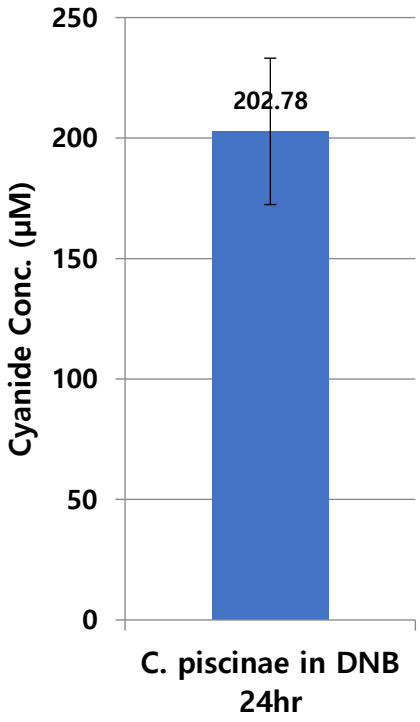


Figure 2.5. Average cyanide concentration produced by *C. piscinae,* cultured overnight in DNB, 15ml cultured in 50ml of Falcon tube.

With a method based on [46], it was continuously confirmed that production of cyanide by *C. piscinae* was approximately 200μM. Cyanide is one of the secondary metabolites, so of course, it could easily change by culture condition including container, media, temperature, and so forth. In our test, culturing condition was constantly maintained and every cyanide concentration used in the experiment was measured to check if the concentration is similar as usual or not.

2.4.6 Different concentration of Potassium Cyanide Inhibits HD100 predation

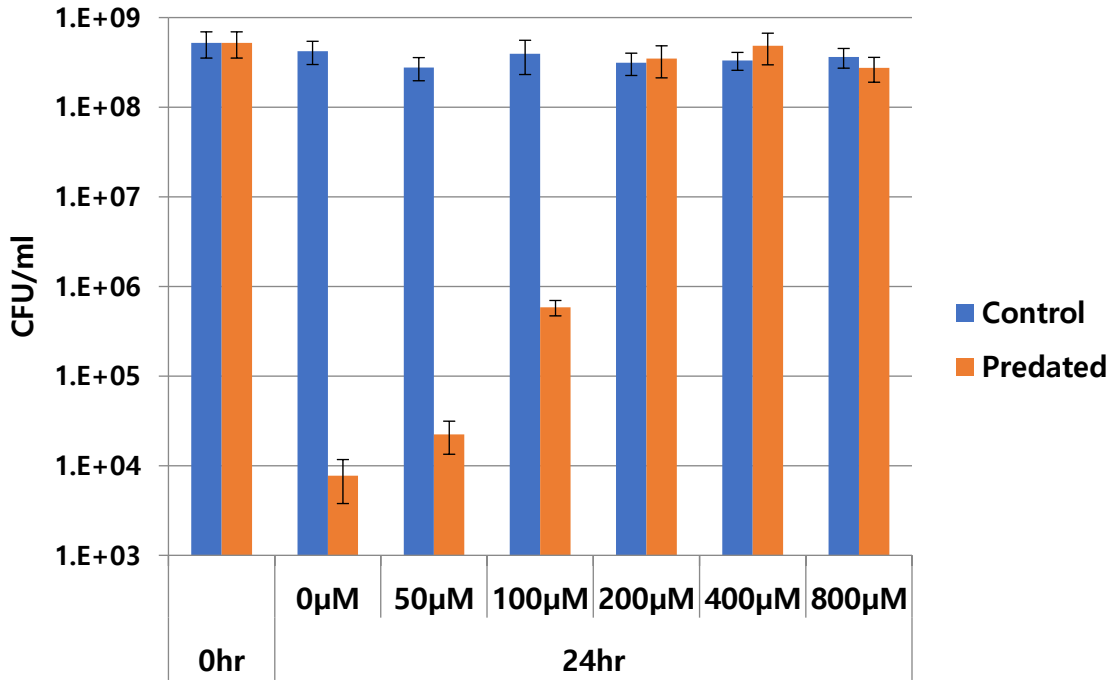


Figure 2.6. Predation with different concentration of cyanide by using KCN. At 0hr and 24hr after adding *B. bacteriovorus* HD100 was sampled. Different concentration of potassium cyanide used in the experiment is written in x-axis in the graph.

Overall preference of CFU of survived *C. piscinae* cells after 24hr predation by *B. bacteriovorus* HD100 indicated that over 200μM of KCN can block *B. bacteriovorus* HD100 predation almost completely within 24hr, and this inhibitory effect was proportionally drops following with serially diluted concentration of KCN. Below 25μM of cyanide, the inhibitory effect was not significant. With this result above, it was certain that cyanide is the main effector in *C. piscinae* supernatant from DNB.

2.5 Discussions

With results of experiments of this chapter, it is sure that *C. piscinae* can inhibit predation of *B. bacteriovorus* HD100 when *C. piscinae* is cultured with nutrient media, in this case, DNB. Its inhibitor exists in the supernatant, and some guessed candidates were set and tested. First, violacein was tested because this substance has an antimicrobial effect on *S. aureus* [48], and it is bisindole, supported by previous research telling indole can reduce motility of *B. bacteriovorus* [42]. Nevertheless, violacein showed no inhibition effect, so another experiment to find a clue of the candidate of effector was done: purging. 3L/min of nitrogen gas was purged into *C. piscinae* DNB supernatant, and it indicated reduced ability of inhibition, meaning that volatile compound is major effector in *C. piscinae* DNB supernatant. This result and the fact that bacteria in *Chromobacterium* produce cyanide in their metabolic pathway [49-51], we assumed that cyanide may major effector in the supernatant. In this context, cyanide concentration in *C. piscinae* supernatant was measured, telling that enough amount of cyanide is produced when *C. piscinae* is cultured in DNB to completely block *B bacteriovorus* HD100 predation in 24hr. The result of dose-dependent predation inhibitory effect of *C. piscinae* DNB supernatant was mimicked in the dose-dependent test with potassium cyanide, by using similar cyanide concentration with the supernatant.

Compounds included in NB (Nutrient Broth, NEOGEN) are 5g of enzymatic digest of gelatin and 3g of beef extract in one liter. DNB (Diluted Nutrient Broth) is ten-fold more diluted NB. It is already studied that cyanide produced by *C. violaceum* is derived from the carbon in methylene and carbon dioxide in carboxyl group of glycine [50], and free glycine concentration. Free glycine concentration in DNB media is calculated as under 10mM at most, and at least about 5mM of glycine is used to make cyanide. It means most of glycine concentration is under cyanide-producing metabolic pathway. Amino acids in nature have various concentration by place and time, but for example of freshwater, free glycine concentration is about 1~2 ug/L in Huron River, Michigan [52]. So, direct comparison of cyanide concentration of *C. piscinae* between in DNB media (in vitro) and in nature is not capable, however, large amount and role of cyanide production have to be thought deeply.

Chapter 3. How Cyanide Changes HD100 Predation with Escherichia coli

3.1 Summary

To know limited steps of *B. bacteriovorus* HD100 predation by cyanide, major two steps of *Bdellovibrio* predation cycle were studied: initial attachment, and intraperiplasmic growth. Experiment for motility was also done for researching about prey finding in the attack phase.

3.2 Introduction

As already stated and studied well, predation cycle of *Bdellovibrio* is divided into some steps including initial attachment to prey in attack phase, and intraperiplasmic growth (followed by septation) with making bdelloplast. Such that, it is important that studying about these two major steps, including motility. Motility of *B. bacteriovorus* has been researched about the function of scouting prey [53] and move to a prey-rich place by rotating a polar flagellum [54-56]. Therefore, prey-finding, invading, and growing inside prey steps were tested in the paper.

3.3 Materials and Methods

3.3.1 Motility test at initial stage of HD100 predation

Experiment method was based on [42]. Overnight cultured of *B. bacteriovorus* HD100 was prepared (liquid cultured at least twice before using in this experiment) and incubated in the absence or presence of 200μM potassium cyanide. It was incubated in a shaking incubator (250 rpm) at 30°C, and samples were taken over time and plated to count the active predatory *B. bacteriovorus* cells. Cell^R (OLYMPUS, USA) microscope was used to take videos (each video was taken 25 sec).

3.3.2 Initial attachment of HD100 predation

Experiment method was based on [42]. Overnight cultured of *B. bacteriovorus* HD100 was prepared (liquid cultured at least twice before using in this experiment), and serially diluted potassium cyanide were made (1M -> 100mM -> 10mM -> 1.6mM -> 800μM -> 400μM -> 200μM -> 100μM -> 50μM) in HEPES.

Overnight cultured *E. coli* MG1655 was centrifuged (15min, 1878 rcf) resuspended with HEPES buffer up to OD600~3.0, and overnight cultured *B. bacteriovorus* HD100 was also centrifuged at 4'C (10min, 16100 rcf) to make 2X concentrated HD100.

Experiments were done with 15ml Falcon tubes, to contain 1.8ml of potassium cyanide solutions, 100ul of concentrated *E. coli* MG1655, and 100ul of concentrated *B. bacteriovorus* HD100. MOI was approximately 1. It was incubated in a shaking incubator (250 rpm) at 30°C. CFU and PFU of tubes were measured at initial time(0h) and 24hr later from incubation.

3.3.3 Intraperiplasmic growth of HD100 with cyanide

Experiment method was based on [42]. Overnight cultured of *B. bacteriovorus* HD100 with VENUS plasmid was prepared (liquid cultured at least twice before using in this experiment), and serially diluted potassium cyanide were made (1M -> 100mM -> 10mM -> 4mM -> 2mM -> 1mM) in HEPES, to be used as 20X potassium cyanide solutions in this experiment.

Overnight cultured *E. coli* MG1655 with RFP plasmid was centrifuged (15min, 1878 rcf) resuspended with HEPES buffer up to OD600~2.0, and overnight cultured *B. bacteriovorus* HD100 was also centrifuged at 4'C (10min, 16100 rcf) to make 2X concentrated HD100.

Experiments were done with 15ml Falcon tubes, to contain 1ml of concentrated *E. coli* MG1655 and 1ml of concentrated *B. bacteriovorus* HD100. MOI was approximately 1. It was incubated in a shaking incubator (250 rpm) at 30°C, and 20X potassium cyanide was added after 1hr incubation to make bdelloplast. Sampling was done at 0hr, 1hr, 3hr, and 6hr from start of incubation. Microscopic images were taken with LSM780, in UNIST.

3.3.4 ATP pool test of HD100 with Cyanide

ENLITEN® ATP assay system bioluminescence detection kit for ATP measurement was used to observe ATP of initial(0h) *B. bacteriovorus* HD100, and ATP of control group added HEPES buffer and the experimental group added 200μM KCN, a similar concentration of *C. piscinae* DNB supernatant after 2hr incubation (250rpm, 30'C).

3.4 Results and discussions

3.4.1 Motility test at initial stage of HD100 predation

By comparing the speed of control group (incubated with HEPES buffer) and experimental group (incubated with 200μM of potassium cyanide) in taken video with IX81, it was clear that each group of *B. bacteriovorus* HD100 have different motility. Control groups showed normal motility, similar with as stated previous paper [42], and experimental group with 200μM of potassium cyanide showed obviously reduced motility, and somehow tumbled. As mentioned in other papers, motility of *B. bacteriovorus* has a role of finding prey [53] and move to prey-rich place by rotating a polar flagellum [54-56]. Such that, this kind of effect of cyanide for *B. bacteriovorus* could be assumed as decrease of prey-finding capacity and ability to move into the prey-rich region.

3.4.2 Initial attachment of HD100 predation

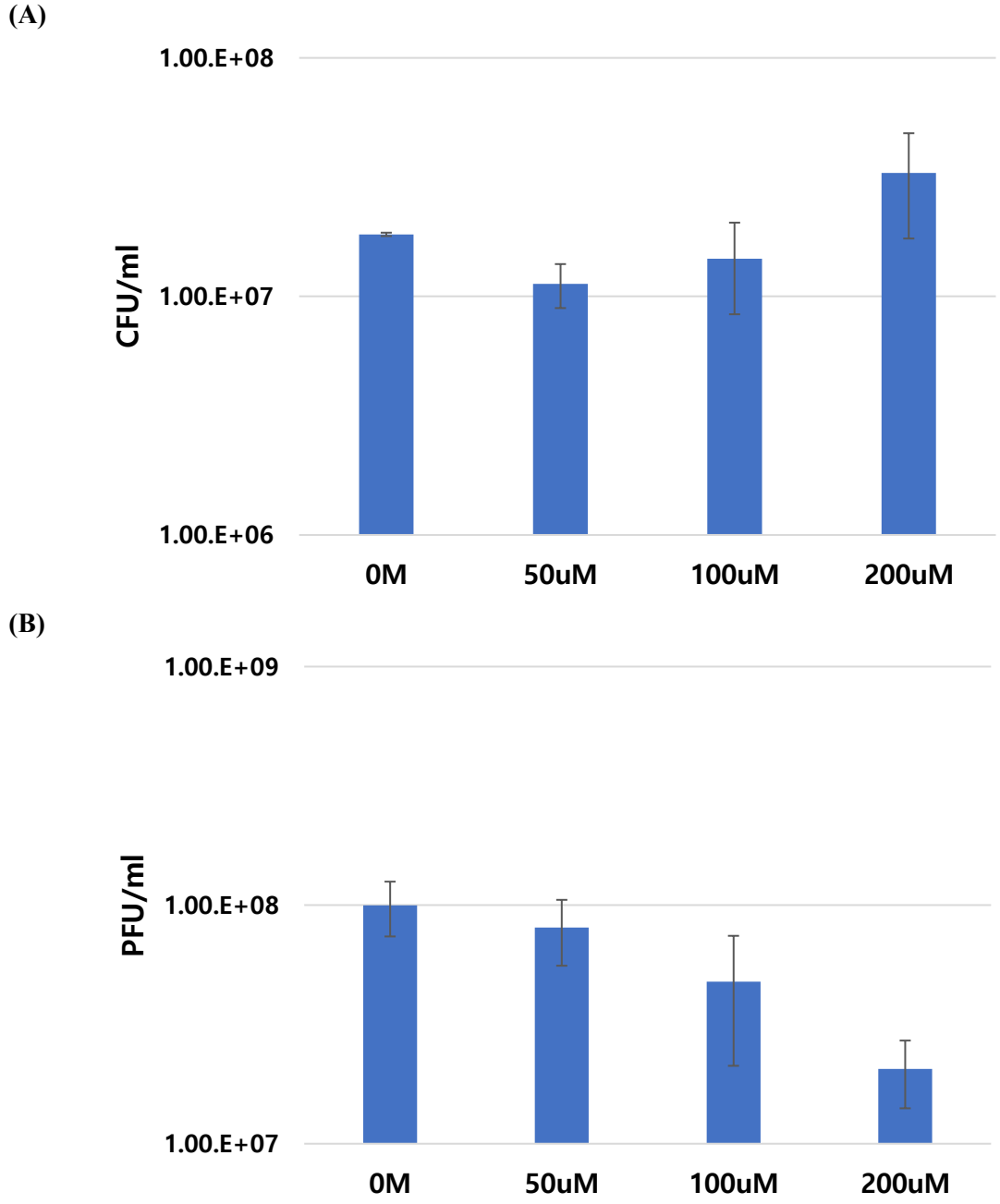


Figure 3.1. Initial attachment of *B. bacteriovorus* HD100 to *E. coli* MG1655 with KCN. (A) is CFU, and (B) is PFU graph after 2 hours of *E. coli* MG1655 predation by *B. bacteriovorus* HD100 inside KCN solution based HEPES buffer. (A) shows increasing preference of CFU, survived *E. coli* MG1655 from BALO predation, proportional to cyanide concentration. (B) presents decreasing preference of PFU, progeny number of *B. bacteriovorus* HD100 by predation, with increasing cyanide concentration.

With increasing cyanide concentration, CFU value of *E. coli* MG1655 was increasing and PFU value of *B. bacteriovorus* HD100 was decreasing in 2hr of predation. It shows the ability of cyanide gave adverse effect for initial attachment, proportional to its concentration. Relating this result and Fig 3.1 with motility test (3.4.1) in this paper, we can know that attack phase of *B. bacteriovorus* HD100 is surely affected by cyanide.

3.4.3 Intraperiplasmic growth of HD100 with cyanide

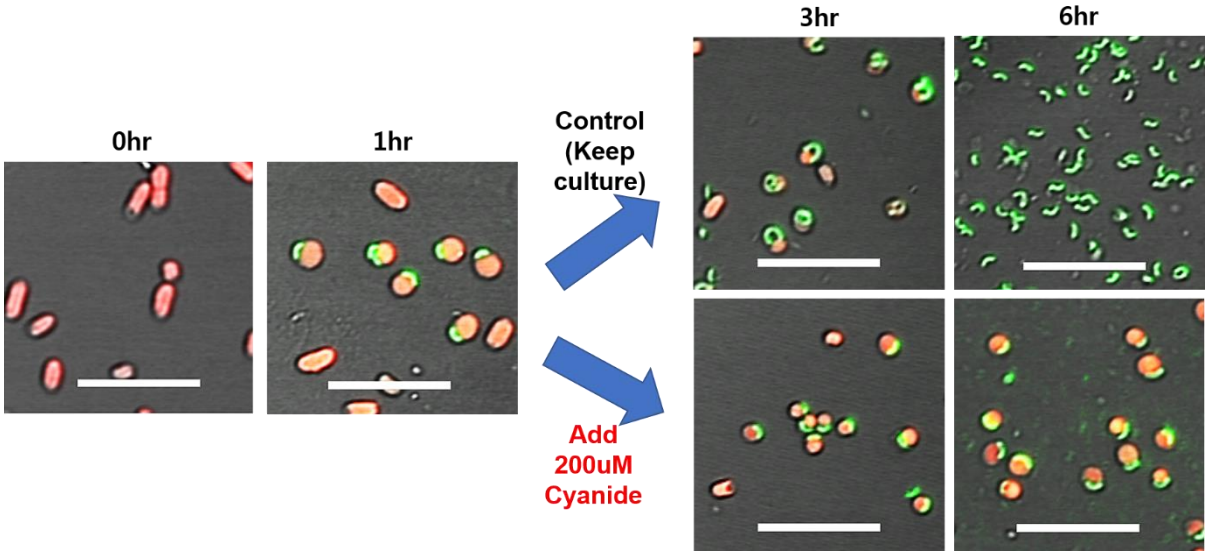


Figure 3.2. Intraperiplasmic growth of *B. bacteriovorus* HD100 with potassium cyanide. Prey is *E. coli* MG1655 with RFP plasmid (shown as red), and predator is *B. bacteriovorus* HD100 with VENUS plasmid (shown as green). The size of scale bar is 10μm. The picture at 0hr incubation shows prey only, and at 1hr just presents well-formed bdelloplasts by HD100. Then potassium cyanide was added for experimental group to make final 200μM concentration, and the same volume of HEPES buffer was put in the control group.

As for showing in Fig 3.1, the control group showed elongated predator inside of host at 3hr, and lysed-out predator could be seen at 6hr incubation picture. By contrast, experimental group with 200μM of potassium cyanide showed very delayed growth-actually almost no change of growth both at 3hr and 6hr. With this result, it is assumed that effect of cyanide would give influence on the intraperiplasmic step in predation cycle of *Bdellovibrio*.

3.4.4 ATP pool test of HD100 with Cyanide

With the 2hr exposure of 200μM cyanide with attack phase HD100 without prey cell, ATP level was reduced. The relative percentage of the 2h experimental group compared to 2h control group was 64.17%, although overall ATP measurement showed huge standard deviation. Nevertheless, it means ATP of HD100 quite decreased with 200μM of cyanide.

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