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Quinolone Signals Related to *Pseudomonas* Quinolone Signal-Quorum Sensing Inhibits the Predatory Activity of *Bdellovibrio bacteriovorus*

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Bdellovibrio bacteriovorus is one of the predatory bacteria; therefore, it can act as a novel "living antibiotic," unlike the current antibiotics. Here the predation of *Escherichia coli* by *B. bacteriovorus* was inhibited in the presence of *Pseudomonas aeruginosa*. This study investigated whether *P. aeruginosa*-induced predation inhibition is associated with bacterial quorum sensing (QS). Each *las*, *rhl*, or *pqs* QS mutant in *P. aeruginosa* was used to check the predatory activity of *E. coli* cells using *B. bacteriovorus*. As a result, the predatory activity of *B. bacteriovorus* increased in a mutant *pqs* QS system, whereas wild-type PA14 inhibited the predatory activity. Moreover, the addition of 4-hydroxy-2-heptylquinoline (HHQ) or the analog triggered the low predatory activity of *B. bacteriovorus* and killed *B. bacteriovorus* cells. Therefore, a defensive action of *P. aeruginosa* against *B. bacteriovorus* is activated by the *pqs* QS system, which produces some quinolone compounds such as HHQ.

Keywords: inhibition factors, *Escherichia coli*, quorum sensing, *Pseudomonas* quinolone signal, *Pseudomonas aeruginosa*, *Bdellovibrio bacteriovorus*, predatory bacteria

INTRODUCTION

Bdellovibrio bacteriovorus is a small-sized Gram-negative bacterium belonging to the group *Bdellovibrio* and like organisms (BALOs). It is one of the obligate predatory bacteria which prey another Gram-negative bacterium (Stolp and Starr, 1963). Although *B. bacteriovorus* cannot prey upon Gram-positive bacterial species, it is known to be able to degrade the biofilm formed by Gram-negative bacteria and Gram-positive ones (Kadouri and O'Toole, 2005; Dashiff et al., 2011; Im et al., 2018). *B. bacteriovorus* also has a competitive inhibition against *Staphylococcus aureus* (Im et al., 2018). BALOs exist in a diverse environment such as waste sewage sludge (Koval et al., 2013), river (Hobley et al., 2012), sea (Pineiro et al., 2007; Chen et al., 2015; Tang et al., 2020), and various habitats on the ground (Jurkevitch et al., 2000). *B. bacteriovorus* usually grows through the invasion, digestion, and lysis of other Gram-negative bacteria as host cells; then, it again swims to

find other new host cells, predate, and replicate further (Pérez et al., 2016). In the predation for Gram-negative bacteria, *B. bacteriovorus* takes 2–4 h to complete all the processes, including the attachment using type IVa pili (Evans et al., 2007; Mahmoud and Koval, 2010), the modification of peptidoglycan of host cells (Stolp and Starr, 1965; Kuru et al., 2017), the digestion, and the lysis, which some enzymes can handle as obtained from *B. bacteriovorus* (Thomashow and Rittenberg, 1978a,b; Lerner et al., 2012). Hence, *B. bacteriovorus* has the potential to be applied in many fields—for example, *B. bacteriovorus* may have a certain potential to kill multidrug-resistant bacteria (Iebba et al., 2014), remove harmful bacteria on food (Fratamico and Cooke, 1996), decrease waste sewage sludge (Semblante et al., 2017), and disperse biofilm on the pipes in wastewater treatment plants (Feng et al., 2016). To date, *B. bacteriovorus* is expected to be available for cystic fibrosis patients with chronic infections (Sockett and Lambert, 2004; Shatzkes et al., 2015) caused by *Pseudomonas aeruginosa* which is known as one of the critical strains of multidrug-resistant bacteria because *B. bacteriovorus* was discovered to prey on *P. aeruginosa* by some researchers (Stolp and Starr, 1963; Shanks et al., 2013; Iebba et al., 2014). However, other researchers also reported that *P. aeruginosa* is not preyed upon by *B. bacteriovorus* (Mukherjee et al., 2015). Thus, the predatory performance of *B. bacteriovorus* to *P. aeruginosa* strains is inconsistent; however, the reason is still unknown.

Most bacteria in nature do not live independently but communicate with each other through extracellular signal molecules to control their collective behavior. These signal molecules are mechanisms released into the environment and respond after they are taken up by the surrounding cells (Fuqua et al., 2001; Miller and Bassler, 2001; Withers et al., 2001). Quorum sensing (QS), one of the most essential systems for regulating bacterial functions, is a typical communication system used by many bacterial species to activate or repress a large number of genes, which form the QS regulon. Changes in their expression levels are achieved once the signal molecules reach a given threshold (Fuqua et al., 1994). It controls bacterial functions such as biofilm formation, resistance to acid stress, motility, competence, and morphological modifications (Huang et al., 2016; Johansen and Jespersen, 2017; de Almeida et al., 2018). Gram-negative bacteria use small diffusible molecules called autoinducers such as indole (Lee et al., 2015), acyl-homoserine lactones, autoinducer-2 (Galloway et al., 2011), and alkyl-quinolone (Pesci et al., 1999) among others—for example, *P. aeruginosa* also regulates the gene expression of some virulence factors by using four QS systems directly or indirectly: *las* regulation system, *rhl* regulation system, *pqs* regulation system, and *iqs* regulation system (Winson et al., 1995; Latifi et al., 1996; Pesci et al., 1997, 1999; McKnight et al., 2000; Gallagher et al., 2002; Kiratisin et al., 2002; Déziel et al., 2004; Xiao et al., 2006; Lee et al., 2013). *P. aeruginosa* uses 3-oxo-C_{12}-homoserine lactone for the *las* QS system, C_4-homoserine lactone for the *rhl* QS system, and integrated QS signal [IQS; 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde]. In particular, the *pqs* QS system is known as the most complicated one among the QS systems of *P. aeruginosa* since the system has three kinds of quinolone compounds: *Pseudomonas* quinolone signals [PQS; 2-heptyl-3-hydroxy-4(1H)-quinolone], 4-hydroxy-2-heptylquinolone (HHQ), and 2-heptyl-4-quinolinol-1-oxide (HQNO). Thus, all the QS signal molecules have the function of promoting or inhibiting other bacteria, fungi, protozoa, and nematodes (Fuqua et al., 1994; Burgess et al., 1999; Dubuis et al., 2007). Changes in the expression of autoinducer and QS-controlled compounds can also affect not only bacterial community dynamics but also host responses during infection. It has been reported that various QS regulatory molecules, such as acyl-homoserine lactones, quinolones, and phenazines, can interact with host cells and thereby affect various responses, including immune regulation (Liu et al., 2015). However, *E. coli* strains, including *E. coli* O157:H7 (Page et al., 2015), use indole as one of the signal molecules (Wang et al., 2001), and Dwidar et al. (2015) investigated that a high concentration (2 mM) of indole inhibits the predation of *E. coli* by *B. bacteriovorus*. Since 0.25–1.1 mM indole was detected in human feces (Karlin et al., 1985; Zuccato et al., 1993), indole may help *E. coli* to inactivate the predatory activity of *B. bacteriovorus* if they grow together. Thus, we hypothesized that the QS systems of Gram-negative bacteria might influence the *B. bacteriovorus* cells negatively.

Although the effect of indole on the predatory activity of *B. bacteriovorus* was investigated, there are no reports on how other QS molecules of Gram-negative bacteria influence the activity of *B. bacteriovorus*. In this study, the relationship between the QS systems of *P. aeruginosa* and the predatory activities of *B. bacteriovorus* was investigated.

MATERIALS AND METHODS

Chemicals

The following compounds, 2-heptyl-3-hydroxy-4(1H)-quinolone (PQS; *Pseudomonas* quinolone signal), 4-hydroxy-2-heptylquinolone (HHQ), and 2-amino-6-chlorobenzoic acid (CABA), which is a *pqs* QS inhibitor, were purchased from Sigma-Aldrich Japan (Tokyo, Japan), and 2-heptyl-4-quinolinol-1-oxide (HQNO) was purchased from Cayman Chemical Company (Michigan, United States). As one of the quinolone analogs, nalidixic acid was also purchased from FUJIFILM Wako Pure Chemical Corporation, Ltd. (Osaka, Japan). PQS and HHQ were stocked in dimethyl sulfoxide (DMSO) to make an 80-mM standard solution. HQNO, nalidixic acid, and CABA were stocked in DMSO to be 8-mM, 16-mM, and 1.5-M stock solutions, respectively.

Bacterial Strains

B. bacteriovorus 109J (ATCC 43826), used as a predatory bacterium in this study, was purchased from the ATCC culture collection. The glycerol stock of *B. bacteriovorus* cells was made by mixing a cell culture of *B. bacteriovorus* and an equal volume of 2 × stock solution consisting of 10 g of yeast extract, 100 ml DMSO, 100 ml glycerol, 312 ml of 0.2 M NaH_2PO_4, and 488 ml of 0.2 M Na_2HPO_4 in 1 liter of the total stock solution, and the mixture was stored at −70°C. The *Escherichia coli* strains and *P. aeruginosa* strains used in this study are listed in **Table 1**.

TABLE 1 | Strains used as host cells in this study.

Strains	Genotype	Sources
Escherichia coli K-12 BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$; parental strain for the Keio collection	Datsenko and Wanner, 2000
E. coli K-12 BW25113 *tnaA*	K-12 BW25113 Δ*tnaA* Ω Km^{R}	Baba et al., 2006
Pseudomonas aeruginosa PA14	*P. aeruginosa* wild type	Liberati et al., 2006
P. aeruginosa pqsA	PA14 *pqsA* Ω *Mar2xT7*, Gm^{R}	Liberati et al., 2006
P. aeruginosa pqsH	PA14 *pqsH* Ω *Mar2xT7*, Gm^{R}	Liberati et al., 2006
P. aeruginosa lasI	PA14 *lasI* Ω *Mar2xT7*, Gm^{R}	Liberati et al., 2006
P. aeruginosa rhlI	PA14 *rhlI* Ω *Mar2xT7*, Gm^{R}	Liberati et al., 2006

Km^{R}, kanamycin resistance; Gm^{R}, gentamicin resistance.

Growth Condition and Predation Assay

These strains were initially streaked on Luria Bertani (LB) (Sambrook et al., 1989) agar plates and incubated overnight at 37°C. Then, a single colony of each strain was used for inoculation. For the cell preparation of prey cells, a single colony of *E. coli* and *P. aeruginosa* strains was inoculated into 100 ml of LB medium and aerobically incubated overnight at 37°C and 120 rpm. Mainly *E. coli* cells in the log phase (8–12 h) were used. The overnight culture was centrifuged for 10 min at 7,000 rpm and 4°C to wash the cell pellet using HEPES-based buffer, including 25 mM HEPES and 2 mM $CaCl_2$ (the pH was adjusted to 7.6 using 5 M NaOH). Then, each washed pellet was suspended and adjusted at ∼1.5 or two of OD_{600} by the same HEPES buffer. The final cell suspensions were used for propagating *B. bacteriovorus* cells (when *E. coli* BW25113 was used) and for the predation assays in the experiments. For the predation assay using the double-layer method, as mentioned above, the top agar (3 ml) consisting of *B. bacteriovorus* culture (100 μl) and *E. coli* overnight culture (100 μl) was first poured to the bottom agar plate. After the agar has hardened, 2 μl of bacterial overnight culture and/or 2 μl of standard chemical solution was put on the center of each plate, and these plates were incubated at 30°C for 2–3 days. An inhibition zone (the area where *E. coli* cells were grown) was measured with a ruler at three points per plate. Three independent experiments were carried out.

Preparation of *B. bacteriovorus* Cells and Double-Layer Method

Initially, the purity of *B. bacteriovorus* stock was confirmed by checking the plaque formation in a double-layer method in which 3 ml nutrient broth (NB) top agar (kept warm at 50°C; 8 g/l NB, 0.286 g/l $MgCl_2$, 0.222 g/l $CaCl_2$, and 8 g/l agar in pure water) mixed with 100 μl *B. bacteriovorus* culture and 100 μl of *E. coli* growth culture (at 37°C and 120 rpm for 10 h) was poured to NB agar bottom plates consisting of 10 g/l agar concentration and incubated at 30°C for around a week. A piece of top agar with the plaque of *B. bacteriovorus* was sliced at 1 cm^2 and transferred under sterile conditions to the *E. coli* cell suspension (60 ml). The mixture was incubated at 37°C and 120 rpm for 2–3 days until the cell turbidity, mainly derived from *E. coli* cells, drastically decreased. Then, a pure cell culture of *B. bacteriovorus* was obtained by removing *E. coli* cells from the culture with a 0.45-μm-pore-size membrane filter. The filtrate was used as the cell culture of *B. bacteriovorus* for all the experiments in this study.

Viability Tests of Prey Cells in the Presence of *B. bacteriovorus*

E. coli and *P. aeruginosa* cell suspensions, as mentioned above, were concentrated to OD_{600} of 2.0. The *E. coli* cell suspensions (5 ml) were incubated with or without the inoculation of *B. bacteriovorus* cell culture (167 μl) (multiplicity of infection, MOI = 0.01–0.1). The MOI was calculated by dividing the viable count of *B. bacteriovorus* in a 167-μl cell culture by the viable count of *E. coli* in 5-ml cell suspension at ∼2 of the cell turbidity at OD_{600}. *B. bacteriovorus* cell culture (167 μl) was also inoculated in a mixture of 2.5 ml *E. coli* and *P. aeruginosa* cell suspension. As a control, without *B. bacteriovorus*, the sample with only *E. coli* and *P. aeruginosa* cell suspension was also prepared. These mixtures were incubated at 30°C and 120 rpm for a week. Then, an appropriate serial dilution of tester samples was prepared by using 0.85% sterile NaCl solution. To evaluate the survival of the prey cells, serial dilution was spread on LB agar plates and incubated at 37°C under aerobic conditions overnight. Then, the colonies grown on each plate were counted. The distinction between *E. coli* colonies and *P. aeruginosa* colonies was determined visually based on the shape, size, and color of the colonies. Here the plates with pure cultures of *E. coli* and *P. aeruginosa*, respectively, were prepared as standards. Two or three independent experiments were carried out.

Pyocyanin Extraction

For pyocyanin extraction, a single colony of *P. aeruginosa* PA14 was inoculated in 5 ml of LB medium and incubated overnight at 37°C and 120 rpm. The overnight culture (1.83 ml) was inoculated in 0.25% casein-M9 medium (100 ml) to be 0.05 of the cell turbidity at OD_{600} (2.5 g casein, 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 1.0 g NH_4Cl, 0.5 g NaCl, 2 ml of 1 M $MgSO_4$, and 0.1 ml of 1 M $CaCl_2$ in 1 liter of water; the pH was adjusted to 7 using 5 M NaOH) and incubated for 24 h at 37°C and 180 rpm. Pyocyanin was extracted by using chloroform and 0.2 M HCl (Essar et al., 1990) from the overnight culture (100 ml) of *P. aeruginosa* PA14. The concentration of pyocyanin extracted was determined by measuring the absorbance at 520 nm using a V-530 spectrophotometer (JASCO Corporation, Tokyo, Japan). The molar absorptivity of acidic pyocyanin

($\varepsilon = 2.46\ mM^{-1}\ cm^{-1}$) (O'Malley et al., 2004) was used for the calculation of the concentration. The acidic pyocyanin solution was finally adjusted to pH ∼7 by adding 5 M NaOH and stocked at a concentration of 6.63 mM. The stock solution was filtered with a 0.20-μm membrane filter.

Viability Tests of Predatory Cells in the Presence of Quinolone Compounds

Each solution of quinolone compounds was mixed with *B. bacteriovorus* cell culture (0.5 ml) and HEPES buffer (4.5 ml) to be at a concentration of 80 μM. As a control including the only solvent, 2 μl DMSO was added to the *B. bacteriovorus* mixture in HEPES buffer without any chemical compound. The mixture was incubated at 30°C and 120 rpm. The mixture was collected in 0, 3, and 6 days to measure the number of viable predatory cells. In evaluating the viability of predatory cells, the double-layer method mentioned above was conducted. An appropriate serial dilution of the *B. bacteriovorus* cell culture was used to count the number (30–300) of plaques formed on the agar plates after incubation at 30°C for around a week. Two independent experiments were carried out. For the metabolic activity assay, 180 μl HEPES buffer ($pH = 7.6$) with 80 μM PQS, HHQ, HQNO, and nalidixic acid or 0.1% DMSO as the control condition, 10 μl of *B. bacteriovorus* culture (∼10^6 cells), and 10 μl of color reagent (WST solution: electron mediator reagent = 9:1) purchased from Kishida Chemical Co., Ltd. (Osaka, Japan) were mixed in a well of a 96-well plate. The plate was incubated at 30°C, and the microplate reader measured the aerobic conditions and the absorbance at 450 nm with time (every 12 h up to 48 h). Three independent experiments were carried out.

Quinolone Compound Extraction and Quantification

P. aeruginosa PA14 was cultured in LB medium with 0, 0.75, 1.5, and 3 mM CABA (solvent: DMSO, stock concentration: 1.5 M) by incubation at 37°C and 120 rpm for 10 h. The 5-ml culture was centrifuged at 13,000 rpm for 5 min and filtered using a 0.2-μm-pore-size filter. The supernatant (4 ml) was mixed with 4 ml ethyl acetate (Lépine et al., 2004; Lesic et al., 2007) to extract quinolone compounds. The organic solvent was further evaporated, and the dried fraction of quinolone compounds dissolved in 10 μl DMSO. The final DMSO solution was used to test the growth of *B. bacteriovorus*. Then, 2 μl of DMSO solution containing quinolone compounds or 100% DMSO as a control was put on the center of each plate with the cells of *B. bacteriovorus* and *E. coli* in the top agar as mentioned above. Then, these plates were incubated at 30°C for 3–4 days.

In accordance with **Supplementary Tables 1, 2**, LC-QTOF-MS was performed to quantify each quinolone compound in each fraction. Basically, the conditions and methods were followed as written by Kadokami and Ueno (2019). The ion transitions of each quinolone compound are shown in **Supplementary Table 3**. The calibrations of the concentrations prepared were 1, 5, 10, 50, 100, 500, and 1,000 ng/l of each chemical dissolved in methanol. Three independent experiments were carried out.

Isolation of *Bdellovibrio*-Like Bacteria From River Water

River water was collected from Onga River, Kitakyushu, Japan, and an aliquot of the river water (100 ml) was mixed with *E. coli* cells suspended with HEPES buffer at ∼2 of cell turbidity at OD_{600} (100 ml) to aggregate *B. bacteriovorus* from the river. The mixture was incubated at 30°C and 180 rpm for 2 days. The enriched culture was filtered by a 0.45-μm-pore-size filter, and the filtrate was used for the double-layer method as mentioned above to obtain a single plaque of *Bdellovibrio*-like bacteria. A piece of a top agar plate with the plaque was sliced and added to the *E. coli* cell suspension until the cell turbidity (OD_{600}) decreased (for 3–4 days). A pure culture of a *Bdellovibrio*-like bacterium was obtained by repeating this step three times according to the method of Hobley et al. (2012). Three independent experiments were carried out.

Indole Production Assay

E. coli culture in LB medium was incubated at 30°C and 120 rpm, and the cell turbidity was measured at 600 nm after 24 h. The *E. coli* supernatant (0.5 ml) of the 24-h culture was reacted to 0.2 ml of Kovac's reagent (mixed with 5 g of *p*-dimethylaminobenzaldehyde, 25 ml HCl, and 75 ml pentanol). The organic layer (0.1 ml), Kovac's reagent, was mixed with 0.9 ml HCl-amyl alcohol mixture (30 ml HCl and 90 ml pentanol). The absorbance at 540 nm was measured using a V-530 spectrophotometer (JASCO Corporation, Tokyo, Japan) to determine the indole concentration (Kawamura-Sato et al., 1999). Three independent experiments were carried out.

Statistical Analysis

Compared with the control without any test reagent, each chemical sample was calculated using the means from at least duplicate data ($n = 2$ or 3). Graphics were created using Microsoft Office. According to the Shapiro–Wilk test, each data was normally distributed. Therefore, differences of some results were determined by one-way ANOVA, followed by Tukey's test at a significance level of $p < 0.05$.

RESULTS

Effect of *P. aeruginosa* on the Predation of *E. coli* by *B. bacteriovorus*

First, the effect of *P. aeruginosa* PA14 wild type on the predation of *E. coli* K-12 BW25113 by *B. bacteriovorus* was examined. Under regular conditions in the presence of *E. coli* and *B. bacteriovorus*, the number of *E. coli* cells decreased 1,000–10,000 times by the predation by *B. bacteriovorus* (**Figure 1A**). However, *B. bacteriovorus* could not prey *E. coli* cells in the presence of *P. aeruginosa* PA14 (**Figure 1A**). While the predatory activity of *B. bacteriovorus* was basically observed by checking a clear zone on the agar plate (**Supplementary Figure 1A**), the inhibition of predation by *P. aeruginosa* was seen on NB agar plates after incubation, as indicated in a result wherein *E. coli* cells grew in an area near to where the culture of

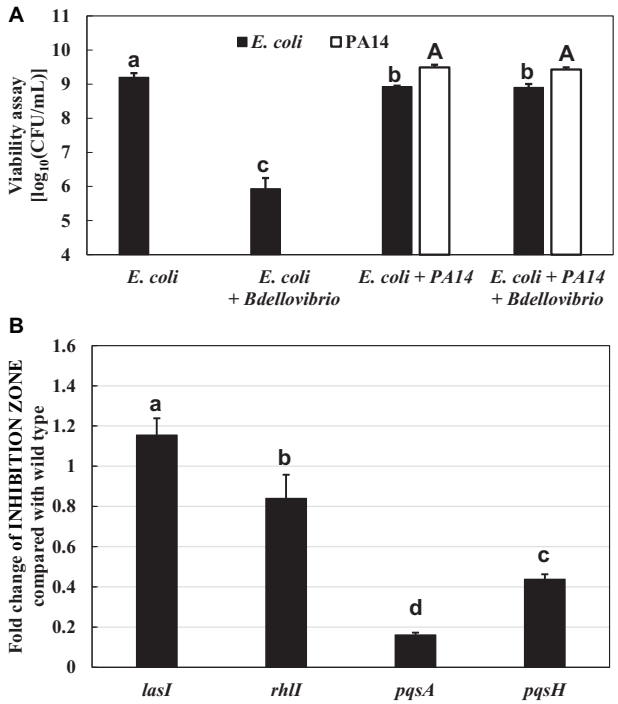

FIGURE 1 | Inhibition of the predatory activity of *Bdellovibrio bacteriovorus* in the presence of *Pseudomonas aeruginosa*. **(A)** Dynamics of viable cells of *Escherichia coli* or/and *P. aeruginosa* PA14 with or without *B. bacteriovorus* 109J. Each cell suspension (initial cell turbidity was adjusted to be ∼2 at OD_{600}) in HEPES buffer was incubated for 72 h at 30°C and 120 rpm under aerobic condition ($n = 2$). **(B)** Effect of *P. aeruginosa* quorum-sensing mutants (*las*, *rhl*, and *pqs*) on the predation plate assays using a mixture of *B. bacteriovorus* 109J and *E. coli* BW25113. In the plate assays using *E. coli* BW25113 as a prey cell, the area of the inhibition zone was compared in the presence of each *P. aeruginosa* strain (*P. aeruginosa* wild type, *lasI* mutant, *rhlI* mutant, *pqsA* mutant, or *pqsH* mutant), $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$).

P. aeruginosa PA14 was spotted on the agar plate (**Supplementary Figure 1B**). Without inoculating *B. bacteriovorus* cells, only the *E. coli* cells were grown on the agar plate (**Supplementary Figure 1C**): note that the reflection of the fluorescent lamp is more diffuse due to the turbidity of *E. coli* growth. Next, the involvement of *P. aeruginosa* QS in the predatory activity of *B. bacteriovorus* was investigated by using each QS mutant (*lasI*, *rhlI*, *pqsA*, or *pqsH*) since these genes are involved in the synthesis of each signal molecule. As shown in **Figure 1B**, whereas a large inhibitory zone was observed in the wild-type strain, *lasI* mutant, and *rhlI* mutant, the zone was reduced when *pqsA* or *pqsH* mutant was tested (**Supplementary Figure 2**, indicated in plate photos). The comparison clearly showed that the two mutants, *pqsA* and *pqsH*, do not have any inhibitory effect on the predation activity of *E. coli* by *B. bacteriovorus* (**Figure 1B**), whereas *lasI* and *rhlI* mutants showed almost the same predation inhibitory activity as the wild-type strain, PA14. Next, when the effect of a complementation assay with QS signal molecules, PQS and HHQ, on the predatory activity by *B. bacteriovorus* was investigated, the addition of PQS at the *pqsH* mutant or HHQ at the *pqsA* and *pqsH* mutants enhanced the inhibitory effect on the predation activity of *B. bacteriovorus* (**Figure 2**). In this experiment, the *pqsA* mutant strain was used as the strain that does not produce both PQS and HHQ, and the *pqsH* mutant strain was used as the strain that produces HHQ but not PQS. The recovery of predatory inhibitory activity in the *pqsA* and *pqsH* mutants was revealed by the size of the inhibition zone in the plate assays in the presence of PQS or HHQ (**Supplementary Figure 3**). Therefore, it was discovered that among the QS systems of *P. aeruginosa*, the *pqs* regulation system plays an essential role in inhibiting the predatory activity of *B. bacteriovorus*. HHQ rather than PQS is also more important to inhibit the predation activity of *B. bacteriovorus* because the inhibitory effect on

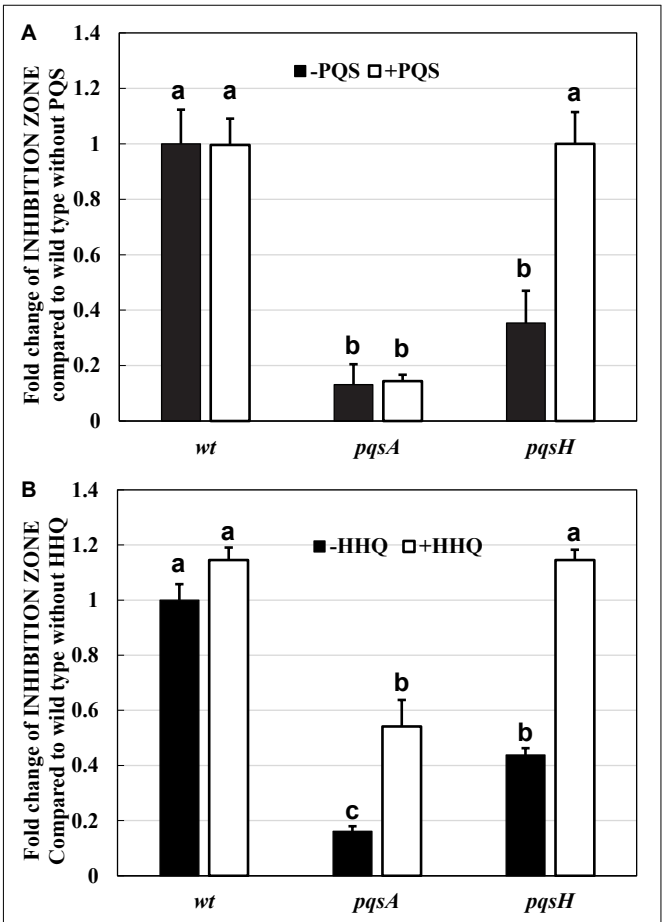

FIGURE 2 | Restoration of the inhibitory effect by two quinolone compounds, PQS **(A)** and HHQ **(B)**, on the predation plate assays using a mixture of *Bdellovibrio bacteriovorus* 109J and *Escherichia coli* BW25113 in the presence of *Pseudomonas aeruginosa* wild type, *pqsA* mutant, or *pqsH* mutant. Each PQS or HHQ standard solution (solvent: DMSO, concentration: 160 nmol) or DMSO solution (control) was placed on the assay plate along with the cell culture of *P. aeruginosa* wild type, *pqsA* mutant, or *pqsH* mutant. All the plates were incubated at 30°C for 4 days. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone. $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$).

the plate assays in the presence of HHQ was greater than that of PQS.

Quinolone Signals Are Essential to the Predation Inhibitory Effect of *B. bacteriovorus*

The next question is whether the predation inhibitory activity to *B. bacteriovorus* is due to each signal molecule (PQS, HHQ, or HQNO) itself or the activation of the *pqs* regulation system by the exogenous addition of PQS, HHQ, or HQNO. First, the predatory activity of *B. bacteriovorus* was evaluated at a final amount of 8, 16, 32, 64, 128, and 160 nmol potential inhibitor by plate assays under the condition that only each *pqs* QS signal molecule (PQS, HHQ, or HQNO) was present without using *P. aeruginosa* culture. As a result, HHQ was the most effective compound in inhibiting the predatory activity of *B. bacteriovorus* (**Figure 3** and **Supplementary Figure 4**). The inhibitory effect was even higher when tested at a final amount of 8, 16, 32, 64, 128, and 160 nmol. Moreover, 100% DMSO addition as the solvent control has no inhibition (**Supplementary Figure 4**). Additionally, the inhibitory effect of the PQS, HHQ, or HQNO was also evaluated at 4, 8, and 16 μM in assays using a liquid culture, by which the predation activity of *B. bacteriovorus* can be evaluated by reducing the turbidity mainly derived from *E. coli* cells. As shown in **Figure 4**, the predatory activity of *B. bacteriovorus* was inhibited according to the concentration of PQS, HHQ, or HQNO tested. The concentrations tested should be reasonable because a similar concentration of PQS, HHQ, and HQNO was detected from the culture of *P. aeruginosa* (Lesic et al., 2007). Particularly, HHQ and HQNO inhibited the activity of *B. bacteriovorus* compared to the control with DMSO. At higher concentrations (20, 40, 80, and 160 μM) of each quinolone compound, the inhibitory effect was also

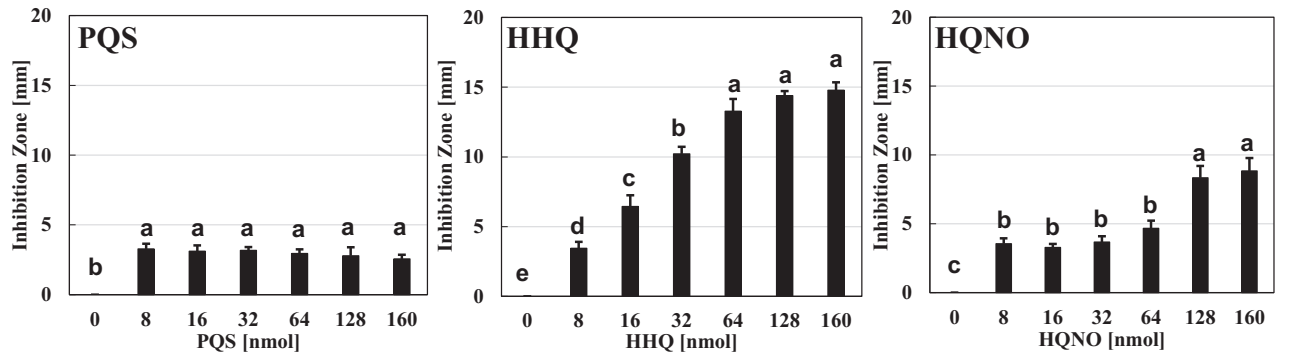

FIGURE 3 | Effect of each quinolone compound itself on the predation plate assays using a mixture of *Bdellovibrio bacteriovorus* 109J and *Escherichia coli* BW25113. The inhibition zone for the predation plate assay using a mixture of *B. bacteriovorus* 109J and *E. coli* BW25113 was compared in the presence of PQS, HHQ, or HQNO compound (0, 8, 16, 32, 64, 128, or 160 nmol) placed on the plate after incubation at 30°C for 4 days. The 0-nmol sample is the control with DMSO alone. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone; HQNO, 2-heptyl-4-quinolinol 1-oxide. $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$).

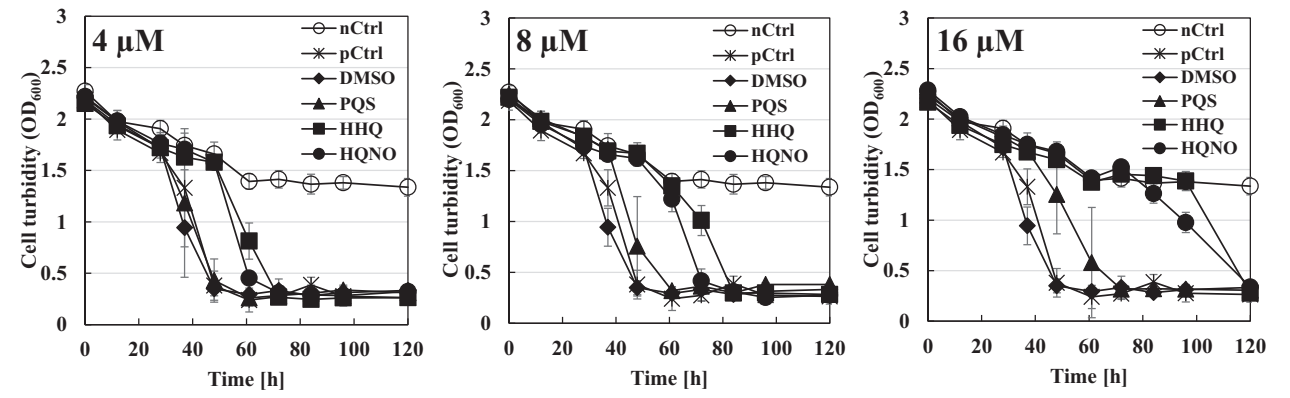

FIGURE 4 | Effect of each quinolone compound itself on the predation liquid assays using a mixture of *Bdellovibrio bacteriovorus* 109J and *Escherichia coli* BW25113. Each cell turbidity of *E. coli* BW25113 ($OD_{600\ nm} \sim 2$) was monitored with time after inoculating the cells of *B. bacteriovorus* under 4 μM **(left)**, 8 μM **(middle)**, or 16 μM **(right)** of each PQS/HHQ/HQNO compound or DMSO which was used as a solvent for the compounds (as the control). "nCtrl" means a sample consisting of only the *E. coli* BW25113 cells in HEPES buffer without any compound, whereas "pCtrl" means a mixture of *E. coli* cells and *B. bacteriovorus* cells in HEPES buffer without any compound. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone; HQNO, 2-heptyl-4-quinolinol 1-oxide. $n = 3$.

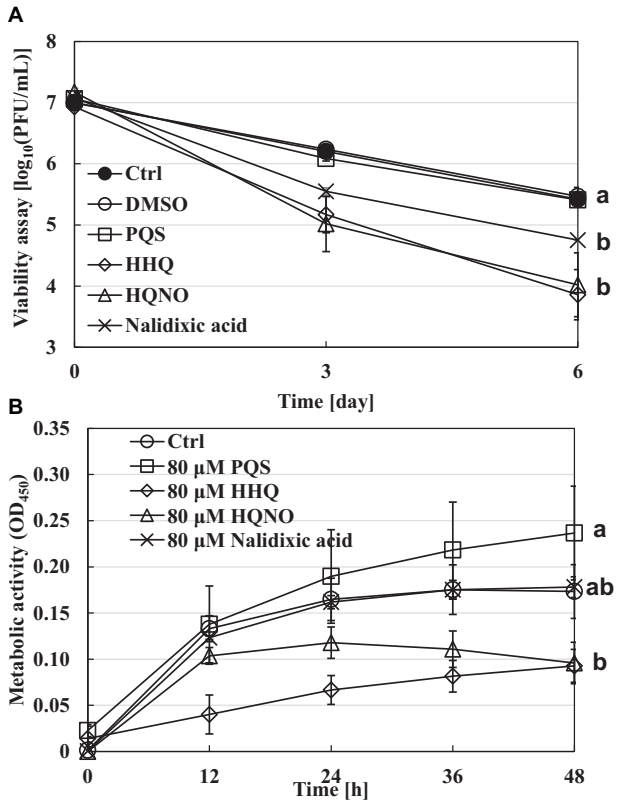

FIGURE 5 | Effect of each quinolone compound itself on the viability of *Bdellovibrio bacteriovorus* cells. **(A)** The dynamics of viable cells of *B. bacteriovorus* in HEPES buffer with or without 80 μM of PQS/HHQ/HQNO/nalidixic acid. The incubation was conducted for 6 days at 30°C and 120 rpm under aerobic condition. $n = 2$. **(B)** The metabolic activity of *B. bacteriovarus* cells with or without 80 μM of PQS, HHQ, HQNO, or nalidixic acid for 48 h at 30°C left to stand in LB medium. "Ctrl" means a mixture of *B. bacteriovorus* cells and DMSO as a control solvent. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone; HQNO, 2-heptyl-4-quinolinol 1-oxide. $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$).

more clearly observed (**Supplementary Figure 5**), as shown in the result wherein complete inhibition of predation activity was found at high concentrations of HQNO (over 20 μM) and HHQ (over 40 μM). Moreover, since pyocyanin is known as a compound produced by the *pqs* QS system (Gallagher et al., 2002), the effect of pyocyanin on the predation activity was examined. Consequently, pyocyanin had no effect on the predation activity of *B. bacteriovorus* (**Supplementary Figure 6**). The results altogether indicate that PQS, HHQ, and HQNO produced by *P. aeruginosa* are essential compounds that inhibit the predation activity of *B. bacteriovorus* directly.

Effect of Quinolone Compounds on *B. bacteriovorus* Cells

The next point is how quinolone compounds act directly on *B. bacteriovorus* cells in the absence of prey *E. coli* cells. The viability of *B. bacteriovorus* cells in HEPES buffer was monitored for 6 days with or without the addition of 80 μM of each quinolone compound, including nalidixic acid, one of the quinolone compounds, as a positive control. As a result, the number of *B. bacteriovorus* cells drastically reduced in the presence of HHQ or HQNO (**Figure 5A**). These compounds may affect the electron mediators of *B. bacteriovorus* since HQNO kills cells by flow inhibition of electrons, such as cytochrome bc_1 complex (Hazan et al., 2016). Corroborating evidence was obtained from the results using the WST-8 colorimetric assay kit to evaluate the metabolic activity of *B. bacteriovorus* under HEPES buffer with or without 80 μM of each quinolone signal. As shown in **Figure 5B**, the metabolic activity of *B. bacteriovorus* was also reduced in the presence of HHQ or HQNO. Hence, our results indicate that quinolone signal compounds produced by *P. aeruginosa* act directly to inactivate *B. bacteriovorus* cells.

Additionally, it was reported that indole represses the predatory activity of *B. bacteriovorus* (Dwidar et al., 2015); it was tested whether *E. coli* can produce indole from several quinolone compounds derived from *P. aeruginosa*. **Supplementary Figure 7** shows the indole production in *E. coli* in the presence of PQS, HHQ, or HQNO. As a result, there was no difference in the amount of indole produced with or without each quinolone compound. Therefore, it is fairly unlikely that the inhibition of the predatory activity of *B. bacteriovorus* may be caused by indole produced from quinolone compounds.

Relationship Between *pqs* Regulation System and Predation Activity of *B. bacteriovorus*

The relationship between the *pqs* regulation system and the predation activity of *B. bacteriovorus* was further examined using CABA, a *pqs* QS inhibitor (Lesic et al., 2007). **Figure 6A** shows the inhibitory effect of *B. bacteriovorus* at several concentrations (0.75, 1.5, and 3 mM CABA), at which the growth of *P. aeruginosa* was not inhibited (**Supplementary Figure 8**). The results clearly show that the predatory activity of *B. bacteriovorus* was not inhibited according to the increasing concentration of CABA. Additionally, the actual concentration of HHQ in each culture was verified by LC-QTOF-MS analyses (**Figure 6C**). As a result, lower concentrations of HHQ were detected from the cultures at higher concentrations of CABA. A similar tendency was observed for the PQS concentration but not for the HQNO one (**Figures 6B,D**). Because the concentration of HHQ is highly correlated with that of CABA compared to PQS and HQNO, the HHQ produced is a key factor to inhibit the predation activity of *B. bacteriovorus*.

Effect of Quinolone Compounds on Other *B. bacteriovorus* Isolates

Another question is whether the inhibitory effect of *B. bacteriovorus* by the quinolone compounds occurs only in *B. bacteriovorus* strain 109J that has been used in this study. To answer the question, two strains of *Bdellovibrio*-like bacteria were isolated from a river sample and similarly evaluated for their effect on quinolone compounds. First, 16S ribosomal RNA gene homology analysis was conducted on these two strains, and it was

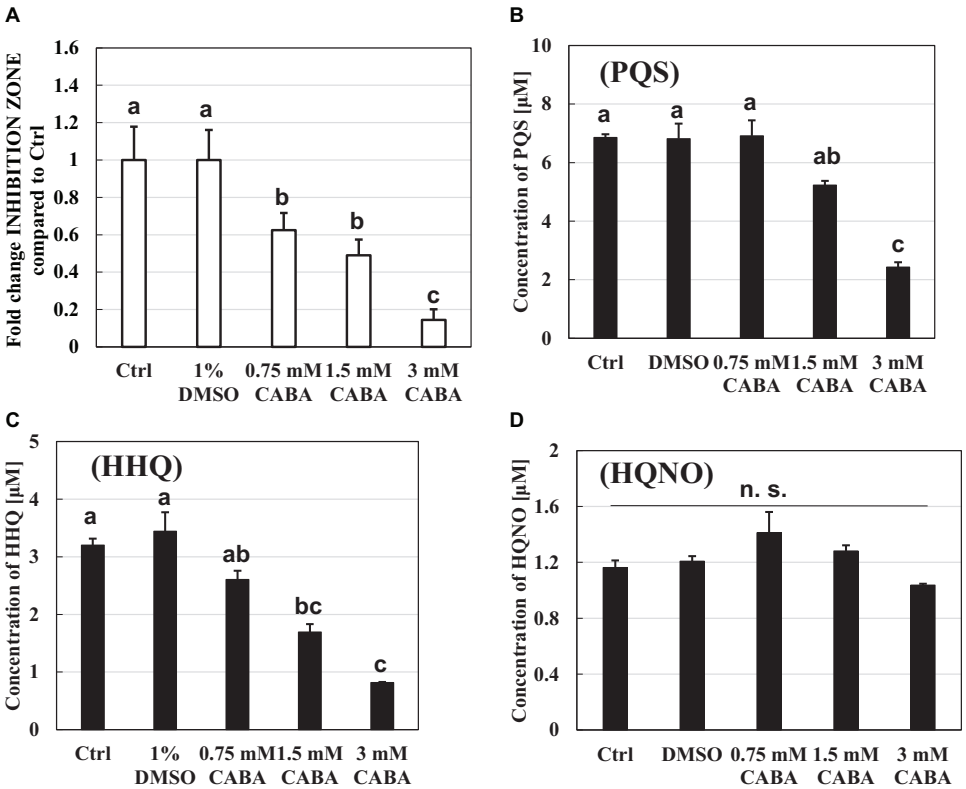

FIGURE 6 | Effect of a *pqs* quorum-sensing inhibitor, 2-amino-6-chlorobenzoic acid (CABA), on the predation inhibition by *Pseudomonas aeruginosa* and the production of PQS, HHQ, or HQNO. **(A)** The reduction of predation inhibitory effect in the presence of CABA. The predation plate assays using a mixture of *Bdellovibrio bacteriovorus* 109J and *Escherichia coli* BW25113 were conducted at 30°C for 3 days in the presence of quinolone compounds extracted from the culture of *P. aeruginosa* wild type containing 0, 0.75, 1.5, or 3 mM of CABA. **(B)** PQS, **(C)** HHQ, and **(D)** HQNO concentration in the extracts of bacterial culture of *P. aeruginosa* PA14 after incubation at 37°C and 120 rpm for 10 h under LB medium with 0, 0.75, 1.5, or 3 mM of CABA. The concentration of HHQ extracted was determined by LC-QTOF-MS. Ctrl and 1% DMSO mean that each sample from the culture uses only LB medium or uses LB with 1% DMSO as solvent control. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone; HQNO, 2-heptyl-4-quinolinol 1-oxide. $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$). $n = 3$. n. s., not significant.

discovered that both strains had 99% homology (TAE1: 99.36%, TAE2: 99.79%) with the *B. bacteriovorus* strain 109J complete sequence. Second, the two *B. bacteriovorus* strains were also inhibited in the presence of quinolone compounds (**Figure 7**). No inhibition zone is observed in the control condition with DMSO alone, as shown in **Supplementary Figure 4**. In both strains, HHQ showed the highest predation inhibitory effect. These results support the above-mentioned point that HHQ should be a key compound to inhibit the predation activity of *B. bacteriovorus*.

Effect of *P. aeruginosa* Mutant Strains That Do Not Produce Quinolone Compounds on the Predation by *B. bacteriovorus*

Considering the previous results, it might be thought that *B. bacteriovorus* can prey on *P. aeruginosa* mutant strains that do not produce quinolone compounds. However, *P. aeruginosa* (*pqsA* or *pqsH* mutant) with a small inhibition zone in **Figure 1B** was not preyed upon by *B. bacteriovorus* when *P. aeruginosa* cell suspension was added to *B. bacteriovorus* (**Supplementary Figure 9**). For *P. aeruginosa* to be preyed upon by *B. bacteriovorus*, it was considered that not only quinolone compounds but also other predation inhibitors had to be avoided.

Next, it was investigated whether it would be possible for *B. bacteriovorus* to prey upon *E. coli* in the presence of *P. aeruginosa* mutant strains, which do not produce quinolone compounds. As a result, *B. bacteriovorus* was unable to prey upon *E. coli* even in the presence of *P. aeruginosa* mutant strains (*pqsA* or *pqsH*) with a small inhibition zone, as shown in **Figure 1B** (**Supplementary Figure 10**).

These results suggest that quinolone compounds are not the only factor inhibiting the predatory activity of *B. bacteriovorus* but that there are other factors.

DISCUSSION

The reason for the presence or absence of predatory activity against *P. aeruginosa* by *B. bacteriovorus* has still been a big mystery to date. *P. aeruginosa*, a Gram-negative bacterium like

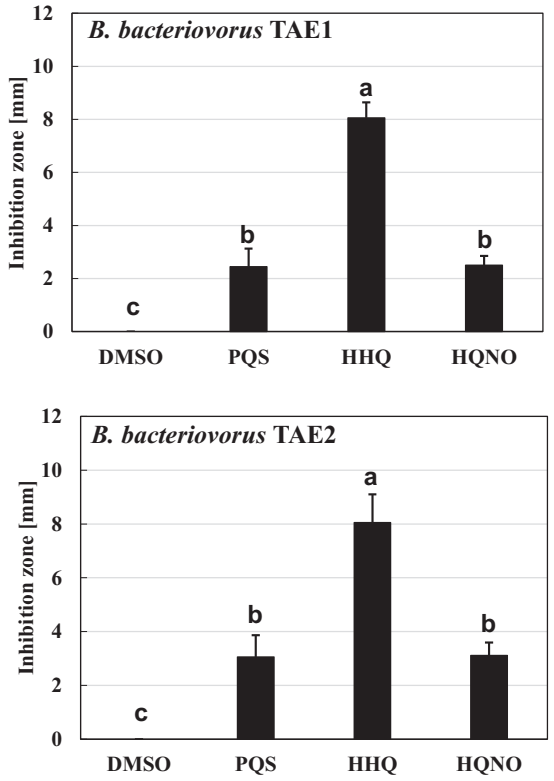

FIGURE 7 | The effect of quinolone compounds on two environmental isolates of *Bdellovibrio bacteriovorus*. The predation plate assay was conducted using two isolates (TAE1 and TAE2) of *B. bacteriovorus*. The inhibition zone for the predation plate assay uses a mixture of each *B. bacteriovorus* isolate, and *Escherichia coli* BW25113 was compared in the presence of PQS, HHQ, or HQNO compound (16 nmol) placed on the plate after incubation at 30°C for 4 days. The DMSO sample is the solvent control with DMSO alone. PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)-quinolone; HHQ, 2-heptyl-4-quinolone; HQNO, 2-heptyl-4-quinolinol 1-oxide. $n = 3$. The value among each sample with different letters is significantly different ($p < 0.05$).

E. coli, has been reported to be preyed upon by *B. bacteriovorus* (Stolp and Starr, 1963; Shanks et al., 2013; Iebba et al., 2014; Pérez et al., 2016), while other researchers have reported such to be not preyed upon (Mukherjee et al., 2015). Since the different behavior regarding predatory activity was considered to be because of the barrier caused by *P. aeruginosa* rather than the property of *B. bacteriovorus* itself, we first investigated the relationship between each QS system derived from *P. aeruginosa* and the predatory activity of *B. bacteirovorus* in this study to narrow down the virulence factors produced by *P. aeruginosa* (Winson et al., 1995; Latifi et al., 1996; Pesci et al., 1997, 1999; McKnight et al., 2000; Gallagher et al., 2002; Kiratisin et al., 2002; Déziel et al., 2004; Xiao et al., 2006; Lee et al., 2013) against *B. bacteriovorus*. These results clearly show that quinolone compounds derived from *P. aeruginosa* negatively affect the predation of *E. coli* by *B. bacteriovorus* (**Figures 1**, **2**, **3**). For the complementary experiments shown in **Figure 2**, the *pqsA* mutant strain, which lacked the first step in the PQS synthesis pathway (Gallagher et al., 2002; Coleman et al., 2008), was not complemented by exogenous PQS since it could not produce HHQ, but the *pqsH* mutant strain, which lacked the conversion gene of HHQ to PQS at the last step in the PQS synthesis pathway (Gallagher et al., 2002; Déziel et al., 2004; Dubern and Diggle, 2008; Schertzer et al., 2009), was complemented by exogenous PQS since it could upregulate PQS synthesis (Xiao et al., 2006) and produce HHQ. However, it is considered to have been complemented in the *pqsH* mutant strain with exogenous HHQ since *pqsA* is upregulated by PqsR receiving HHQ (Rampioni et al., 2016). However, for the *pqsA* mutant strain, the size of the inhibition zone may be due to the exogenous HHQ itself, as shown in **Figure 3**, rather than complementation. Additionally, among the quinolone compounds, HHQ and HQNO directly inactivated the activity of *B. bacteriovorus* (**Figures 4**, **5**). In general, similar phenomena likely occur in nature since *P. aeruginosa* produces 4–40 μM PQS, 0.5–4 μM HHQ, and ∼75 μM HQNO in a pure culture system (Lépine et al., 2004; Lesic et al., 2007; Ji et al., 2016). As the inhibitory mechanism, it inhibits the activity of electron mediators such as cytochrome bc_1 complex (Hazan et al., 2016). However, HHQ exhibits bacteriostatic activity in a species-specific manner (Reen et al., 2011), but the detailed mechanism is still unknown. **Figure 4** and **Supplementary Figure 6** suggest that HHQ (over 40 μM) and HQNO (over 20 μM) are inhibited, but HHQ is as effective as or more effective than HQNO at lower concentrations (4–16 μM). Similarly, in the plate experiment (**Figure 3**), only HHQ showed a more significant inhibition zone than PQS at a low number of compounds (16–32 nmol), while not only HHQ but also HQNO showed a larger inhibition zone than PQS at high amounts of compounds (over 64 nmol). Considering the solubility of each compound in liquid conditions and its diffusion on the plate, the inhibitory mechanism of each quinolone compound against *B. bacteriovorus* needs to be clarified in the future. The effects of HHQ and HQNO are almost the same; however, the concentration of HHQ produced is higher than that of HQNO in the actual culture of *P. aeruginosa* (**Figure 6A**). Additionally, the predatory activity of *B. bacteriovorus* was restored in the presence of a *pqs* regulation inhibitor (Lesic et al., 2007), CABA (**Figures 6B,D**). Thus, the inhibition of predatory activity by HHQ was clarified as a barrier mechanism of *P. aeruginosa* against *B. bacteriovorus*. Since HHQ, which had the highest inhibition in the plate assay, was the focus of this study, instrumental analysis was conducted using the *P. aeruginosa* culture after about 10 h. Therefore, it looks like only the HHQ concentration correlated with the size of the inhibition zone. This may also correlate with the degree of inhibition of HQNO when *P. aeruginosa* is incubated for a longer time (data not shown). Since these *pqs* QS signals produced by *P. aeruginosa* are known to act as not only the regulation of gene expression but also an antibiotic for some bacterial species (Reen et al., 2011; Szamosvári et al., 2019), the predation activity assay of *B. bacteriovorus* strains tested may be inactivated as shown in **Figure 7**.

Although the *pqs* regulation system of *P. aeruginosa* is one of the defense mechanisms against *B. bacteriovorus*, this predator was unable to prey on *pqs* mutants that could not produce quinolone compounds such as HHQ (**Supplementary Figure 9**).

The results show that *P. aeruginosa* may have another mechanism that evades the attack of *B. bacteriovorus*. In the predation evaluation by plate assays, the inhibition zone of *pqs* mutants was small, suggesting a different inhibitory mechanism other than extracellular secretory substances. Further research is needed to elucidate the mechanism of the presence or absence of predatory activity of *B. bacteriovorus* against *P. aeruginosa*.

So far, it has been reported that indole, which is one of the QS molecules, and cyanide inhibits the activity of *B. bacteriovorus* (Dwidar et al., 2015; Mun et al., 2017); however, there is no knowledge about its relationship with other QS compounds. Other than that, Varon and Shilo (1981) reported that the predation of *B. bacteriovorus* was inhibited by sodium dodecyl sulfate (SDS). Surfactants, not limited to artificially added SDS, are produced by various microorganisms such as bacteria and yeast. In **Supplementary Figures 9, 10**, in the presence of the quinolone compound non-producing *P. aeruginosa* strain, *B. bacteriovorus* could not prey upon not only *P. aeruginosa* but also *E. coli*. Thus, the strategy should not be to avoid only one of the extracellular inhibitors to allow *B. bacteriovorus* to prey upon *P. aeruginosa*. As a summary of this research, our results showed that quinolone signals related to the QS of *P. aeruginosa*, especially HHQ and HQNO, inhibit the predation activity of *B. bacteriovorus*. Among the quinolone compounds, HHQ was the most effective quinolone compound against *B. bacteriovorus*. It was discovered that quinolone compounds such as HHQ act as bactericidal on *B. bacteriovorus*, unlike indole that suppresses viability without killing it. As shown in the results of this research, the accumulation of knowledge to understand the relationship between predator bacteria and other bacteria is indispensable for the application and usage of *B. bacteriovorus* in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YH, YN, TMo, and KK performed the experiments. TMa, KK, and RG-C supervised the project. YH initially wrote the manuscript. TMa revised the manuscript. All authors designed the study, discussed the results, contributed to the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.722579/full#supplementary-material

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