ACS Synthetic Biology

Letter

Cite This: ACS Synth. Biol. 2017, 6, 2035-2041

pubs.acs.org/synthbio

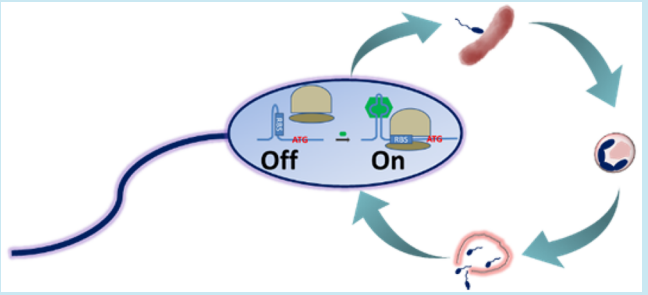
Controlling *Bdellovibrio bacteriovorus* Gene Expression and Predation Using Synthetic Riboswitches

Mohammed Dwidar and Yohei Yokobayashi*

Nucleic Acid Chemistry and Engineering Unit, Okinawa Institute of Science and Technology Graduate University, Onna, Okinawa 904 0495, Japan

Supporting Information

ABSTRACT: *Bdellovibrio bacteriovorus* is a predatory bacterium that feeds on Gram-negative bacteria including a wide range of pathogens and thus has potential applications as a biocontrol agent. Owing to its unique life cycle, however, there are limited tools that enable genetic manipulation of *B. bacteriovorus*. This work describes our first steps toward engineering the predatory bacterium for practical applications by developing basic genetic parts to control gene expression. Specifically, we evaluated four robust promoters that are active during the attack phase of *B. bacteriovorus*. Subsequently, we tested several synthetic riboswitches that have been reported to function in *Escherichia coli*, and identified theophylline-activated riboswitches that function in *B. bacteriovorus*. Finally, we inserted the riboswitch into the bacterial chromosome to regulate expression of the flagellar sigma factor *fliA*, which was previously predicted to be essential for predation, and observed that the engineered strain shows a faster predation kinetics in the presence of theophylline.



KEYWORDS: *Bdellovibrio, predation, riboswitch*

Bdellovibrio bacteriovorus is a predatory bacterium that invades and consumes Gram-negative bacteria to acquire nutrients necessary for its growth and replication.[1] Its life cycle consists of two main phases; the nonreplicating attack phase (AP) in which the predator swims at high speed in the medium searching for a prey, and the intraperiplasmic phase (IP), which takes ∼3−4 h and begins when the predator finds a suitable prey to attach and invade. Shortly after attaching to its prey, the predator penetrates into the prey's periplasm while converting it into a round structure called bdelloplast. Inside this bdelloplast, the predator secretes a cocktail of hydrolases to consume the prey from the inside and utilizes its nutrients to grow and elongate before septation to 3−6 progeny cells, which eventually exit the resource exhausted bdelloplast and proceed to attack other prey cells.[2,3] Due to its unique life cycle, safety,[4,5] and efficacy against a wide range of pathogenic bacteria,[6,7] *B. bacteriovorus* has attracted much attention recently both for its basic biology[8,9] as well as for its potential applications in medicine, industry, and agriculture.[10−16] Consequently, genetic tools that allow control of native and exogenous genes in this unique bacterium should make it more amenable as a microbial chassis for synthetic biology applications. However, *Bdellovibrio*'s dependence on prey bacteria for its survival makes its genetic manipulation more challenging. For example, permanent gene knockout methods routinely used to introduce chromosomal mutations in other bacteria are not always suitable for the *Bdellovibrio* genes that affect the predation behavior, as it may compromise the viability of the mutant cells or necessitate its conversion to a host-independent phenotype, which typically requires mutations in other genetic loci.[9,17,18] Another difficulty is that strong constitutive expression of recombinant or even some native genes in *B. bacteriovorus* may compromise its growth and predation capabilities. Furthermore, the *B. bacteriovorus* research community still lacks a reliable inducible gene expression system to control the timing and the level of gene expression.

Riboswitches are RNA regulatory elements located in noncoding regions of mRNAs that control gene expression in the absence of protein factors.[19−21] A canonical bacterial riboswitch is located in the 5′ untranslated region (UTR). It consists of an aptamer domain that binds a specific small molecule and an expression platform that mediates a structural change that affects premature transcription termination or ribosome binding. Synthetic riboswitches that respond to natural metabolites such as thiamine pyrophosphate (TPP)[22,23] or synthetic compounds such as theophylline[23−25] have been reported. Some of these synthetic riboswitches have been shown to function in diverse bacterial species including *Escherichia coli*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Streptococcus pyogenes*, mycobacteria, cyanobacteria, and others,[24,26,27] suggesting the broad utility of riboswitches across prokaryotes. Moreover, riboswitches are especially useful for

Received: May 25, 2017
Published: August 16, 2017

DOI: 10.1021/acssynbio.7b00171
ACS Synth. Biol. 2017, 6, 2035−2041

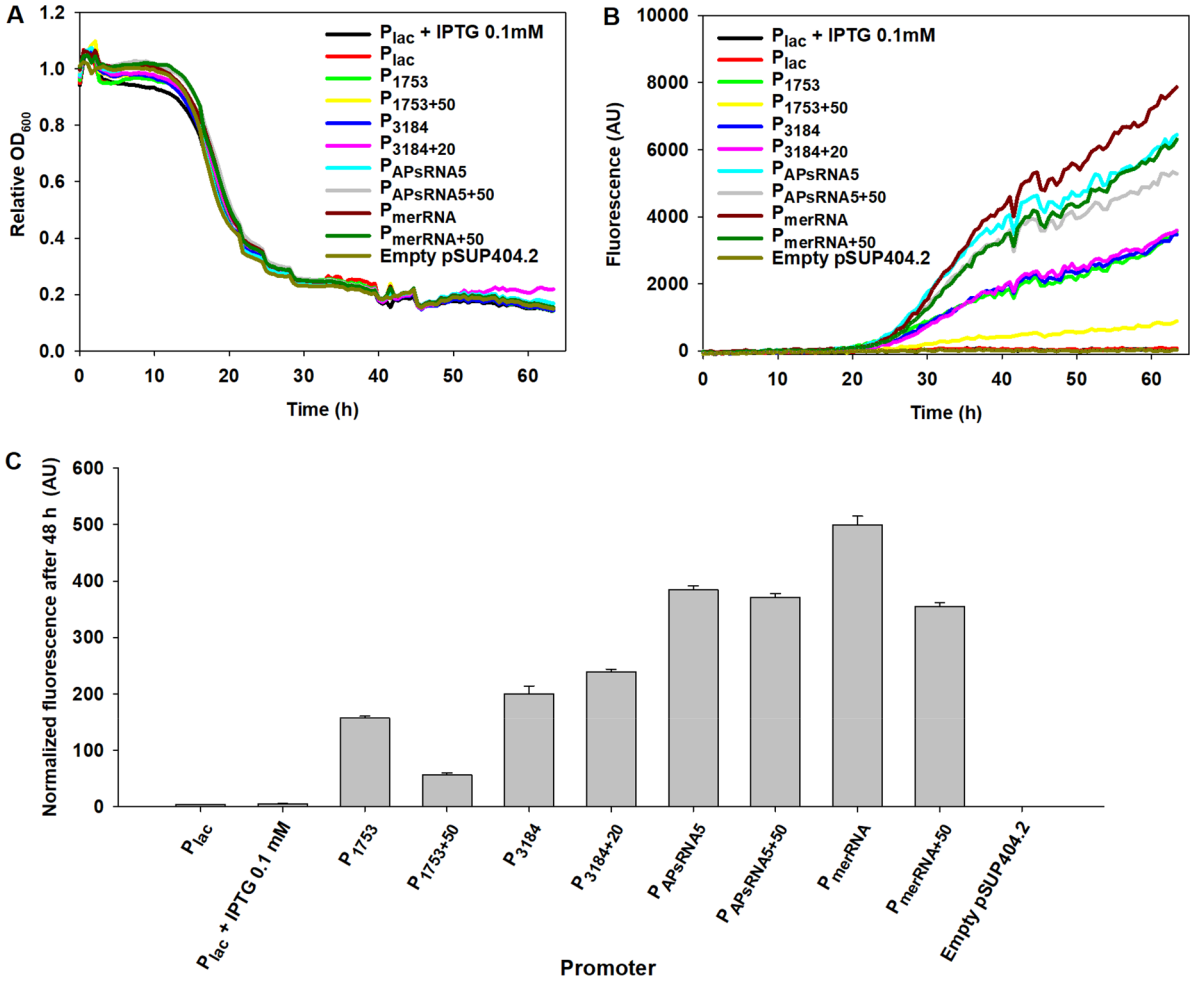


Figure 1. Evaluation of native *B. bacteriovorus* AP promoters. (A) Predation profile of *B. bacteriovorus* harboring a plasmid with mCherry coding sequence under the control of different promoters. Decrease in OD_{600} corresponds to lysis of the prey *E. coli* by *B. bacteriovorus* during predation. Relative OD_{600} values normalized against the control wells without added predators are reported. (B) Time course of mCherry fluorescence of the *B. bacteriovorus* cultures shown in (A). The observed expression profiles are consistent with AP specific promoters. (C) mCherry fluorescence after 48 h culture normalized by *B. bacteriovorus* cell count. The error bars represent standard deviations of three independent cultures.

controlling endogenous gene expression because modification of the associated native promoter is not necessary.

To facilitate fundamental investigations and future applications of *B. bacteriovorus*, we set out to develop basic genetic parts to manipulate gene expression in this unique predatory bacterium. Specifically, we evaluated robust native promoters that are active during the attack phase of the bacterial life cycle, and tested previously described synthetic riboswitches to identify switches that function in *B. bacteriovorus*. Finally, an application of the riboswitch to control an endogenous gene to chemically regulate the predatory behavior of *B. bacteriovorus* is presented.

RESULTS AND DISCUSSION

A previous report on *B. bacteriovorus* used the *E. coli lac* promoter (P_{lac}) for recombinant green fluorescence protein (GFP) expression.[28] Therefore, we initially cloned mCherry under the *lac* promoter control in the plasmid pSUP404.2[28] which was transformed into *B. bacteriovorus* HD100. However, we observed very weak gene expression both in the presence and the absence of IPTG (Figure 1B and 1C). Consequently, the *lac* promoter was replaced with four different native *B. bacteriovorus* promoters P_{1753}, P_{3184}, $P_{APsRNA5}$, and P_{merRNA}, which are associated with the protein coding genes *Bd1753*, *Bd3184*, and small noncoding RNAs APsRNA5, and merRNA, respectively (Table S1). These promoters were previously observed to be highly active during the attack phase of *B. bacteriovorus*.[29] Each of these promoters was fused to mCherry coding sequence with or without the bases downstream of the predicted transcription start site (TSS) because it is sometimes known to affect the rate of transcription.[30,31] As a default, we chose to include 50 bases downstream of the TSS. However, as the native transcript of P_{3184} only contains 20 bases upstream of the start codon, these 20 bases were used in our experiment (Table S1). *B. bacteriovorus* transformed with the plasmids were cultured with prey *E. coli* cells and OD_{600} and mCherry fluorescence were measured over time. As shown in Figure 1A and 1B, mCherry expression driven by these promoters started to increase after 25 h when the decrease in OD_{600} slowed down, confirming that the promoters are more active during the attack phase compared to the intraperiplasmic phase. mCherry fluorescence measurements after 48 h normalized by *B. bacteriovorus* cell count (Figure 1C) demonstrate that the selected attack phase promoters are highly active compared to the *lac* promoter, for example, P_{merRNA} showing 100-fold higher expression compared to P_{lac}.

DOI: 10.1021/acssynbio.7b00171
ACS Synth. Biol. 2017, 6, 2035−2041

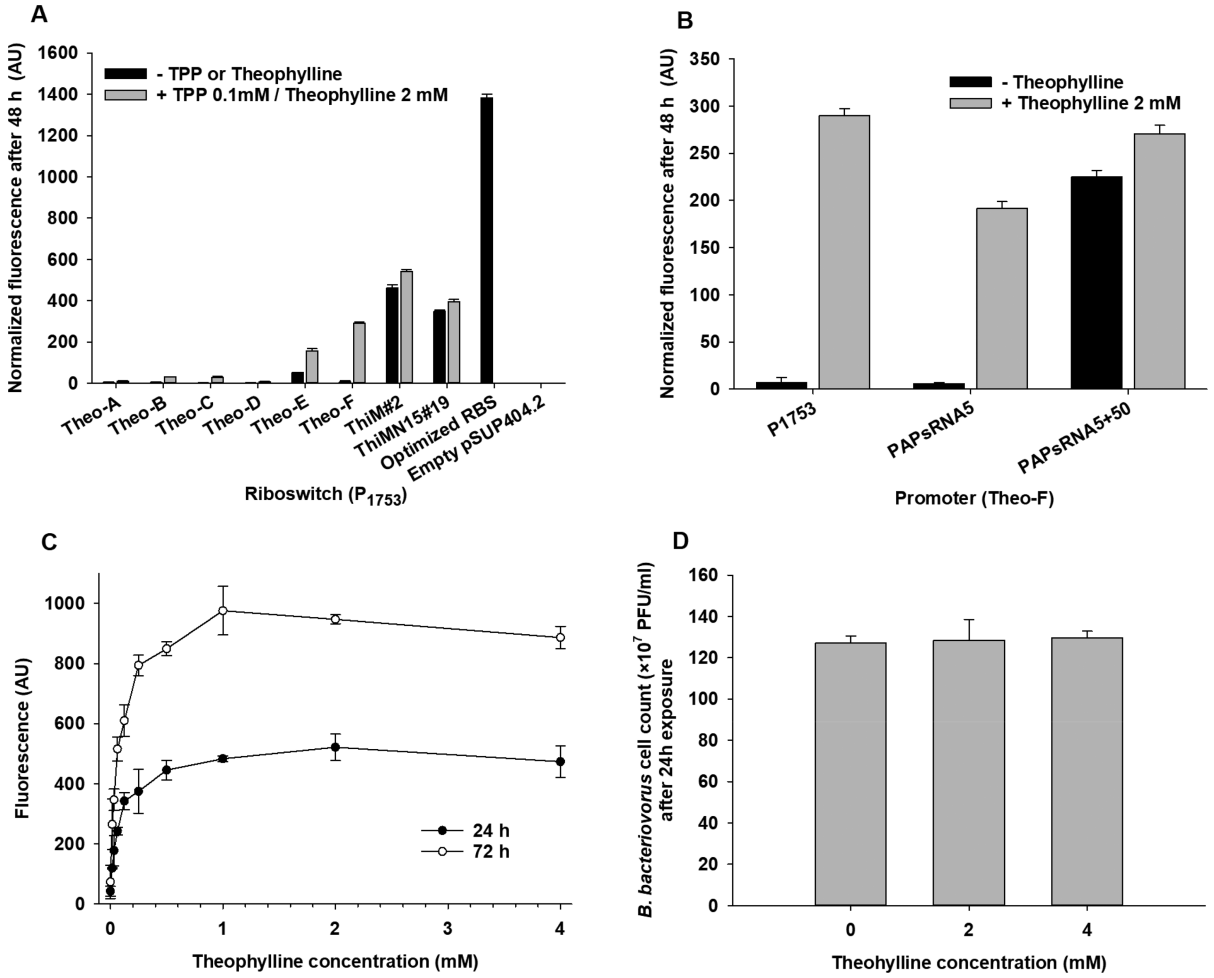


Figure 2. Evaluation of synthetic riboswitches in *B. bacteriovorus*. (A) mCherry fluorescence normalized by cell count of AP *B. bacteriovorus* cultured for 48 h in the presence and absence of the riboswitch ligand. The riboswitches were cloned upstream of the mCherry coding sequence under the control of the promoter P_{1753} in pSUP404.2. (B) Evaluation of Theo-F riboswitch under the control of promoters P_{1753}, $P_{APsRNA5}$, and $P_{APsRNA5+50}$. (C) Dose dependent expression of mCherry (unnormalized) under the control of Theo-F riboswitch expressed from P_{1753} after 24 and 72 h incubation. (D) Viable *B. bacteriovorus* cell count after 24 h incubation with theophylline (2 and 4 mM) as determined by plaque assay. The error bars represent standard deviations of three independent cultures.

The effects of the native sequence downstream of the TSS varied among the promoters. While the effects were minor for P_{3184} and $P_{APsRNA5}$, P_{1753} expressed 64% less mCherry when the 50 native bases downstream of the TSS were included (Figure 1C). It is not clear, however, if the observed difference is due to slower transcription, decreased mRNA stability, or lower translation efficiency. Nevertheless, we identified a set of robust attack phase specific promoters that can be used to drive exogenous gene expression in *B. bacteriovorus*.

Next, we chose one of the promoters above (P_{1753}) to evaluate synthetic riboswitches using mCherry as a reporter gene. We evaluated two TPP responsive riboswitches (ThiM#2, and $ThiMN_{15}$#19) that we previously developed in *E. coli*[22,32] and six theophylline responsive riboswitches (Theo-A, Theo-B, Theo-C, Theo-D, Theo-E, and Theo-F [referred to as Riboswitch E* in the original publication]) from the Gallivan group (Table S2).[24] The riboswitches were placed in the 5′ UTR of mCherry mRNA. mCherry fluorescence was measured after 48 h of culturing attack phase *B. bacteriovorus* cells in the presence and absence of an appropriate ligand and normalized by the cell count.

ThiM#2 and $ThiMN_{15}$#19 riboswitches were evaluated both in the presence of TPP (Figure 2A) and thiamine hydrochloride (Figure S1) because the published genome sequence of *B. bacteriovorus* HD100 does not appear to contain a thiamine kinase (*thiK*) gene that can phosphorylate thiamine to the intermediate thiamine monophosphate (TMP) or pyrophosphokinase for direct conversion into TPP.[33] However, *thiB*, *thiP*, and *thiQ*, which are necessary for importing thiamine as well as TPP,[34] have been annotated (*Bd0593*, *Bd0594*, and *Bd0595* respectively).[2] As shown in Figure 2A and Figure S1, the TPP riboswitches show elevated mCherry expression in the absence of added ligand and do not show appreciable response to TPP or thiamine. This may be due to the presence of high endogenous levels of TMP or TPP produced by *E. coli* and subsequently acquired by *B. bacteriovorus* after predation.

In contrast, some theophylline-responsive riboswitches showed clearly increased mCherry expression in the presence of 2 mM theophylline (Figure 2A). In particular, Theo-F showed low background expression in the absence of theophylline, which increased 40-fold in its presence. Dose-dependence curves of Theo-F show that the riboswitch is fully activated at ∼1 mM theophylline while exhibiting half-maximal

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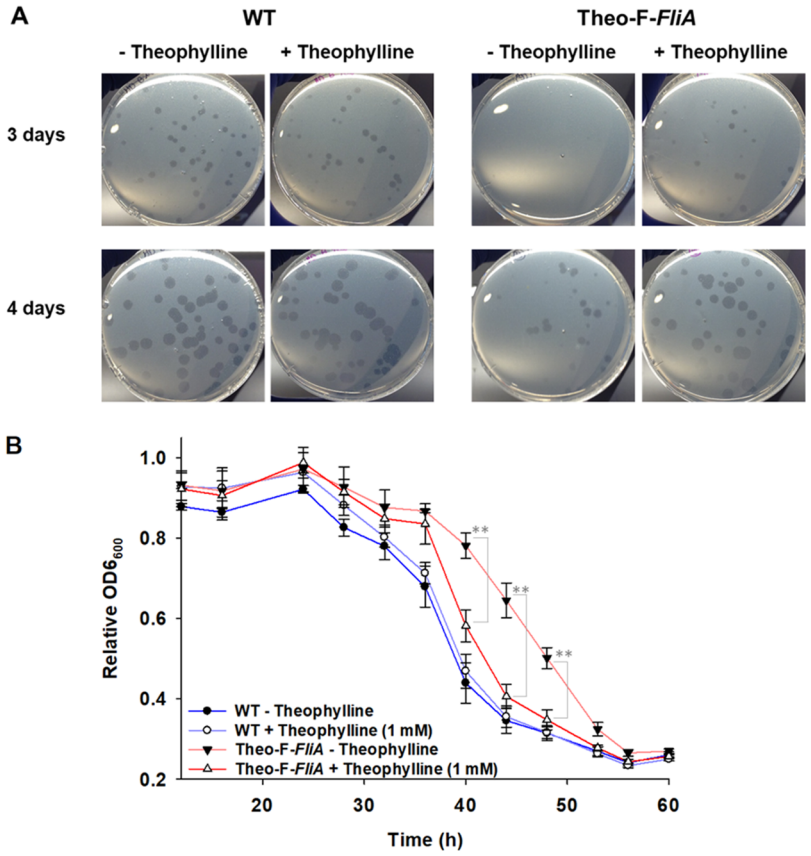

Figure 3. Chromosomal insertion of Theo-F riboswitch to regulate FliA expression. (A) Comparison of plaque formation rates of the wild-type *B. bacteriovorus* HD100 and the Theo-F-*fliA* mutant on double layer agar plates with *E. coli* as a prey, in the presence (1 mM) or absence of theophylline added in the top agar layer. The photos shown are representative of three independent cultures. (B) Comparison of predation rates in liquid culture of the wild-type and Theo-F-*fliA* mutant in the presence (1 mM) or absence of theophylline. Relative OD_{600} values normalized against the control wells without added predators are reported. The error bars represent standard deviations of three independent cultures. (** $P > 0.01$).

response at ~0.06 mM (Figure 2C). It should also be noted that exposure of *B. bacteriovorus* in the attack phase to high concentrations of theophylline (2 and 4 mM) for 24 h did not affect the viability of the bacterium as indicated by the plaque counts on *E. coli* agar plates (Figure 2D). The presence of theophylline at 2 mM, however, caused a slight reduction in the predation rate in liquid cultures (data not shown). Theo-F was further evaluated in the context of $P_{APsRNA5}$ and $P_{APsRNA5+50}$ promoters. Although Theo-F functioned as a riboswitch with $P_{APsRNA5}$, fusion with $P_{APsRNA5+50}$ mostly abolished the riboswitch response (Figure 2B). A plausible explanation is that the extra bases downstream of the TSS somehow interfere with the riboswitch function. This observation underscores the dependence of riboswitch performance on the genetic context. The riboswitches were analyzed by the Riboswitch Calculator, which employs a statistical thermodynamics model to predict riboswitch characteristics.[35] Although the model appears to predict some aspects of the observed riboswitch performance, for example, the low expression levels of Theo-A, B, C, and D, there were also some discrepancies (Figure S2).

With an efficient inducible gene expression system for *B. bacteriovorus* in hand, we explored the possibility of chemically regulating endogenous gene expression by inserting Theo-F into a targeted chromosomal site. In *E. coli* and other related enteric bacteria, the flagellar sigma factor (σ^{28}) FliA controls the expression of class 3 flagellar genes including those that code for the flagellin and the flagellar motor (MotA, and MotB) among other genes.[36] A previous study[29] predicted that FliA (encoded by *Bd3318*) may also control up to 66% of attack phase specific genes in *B. bacteriovorus*. Therefore, we sought to chemically control FliA expression by Theo-F and thus manipulate the predatory behavior of *B. bacteriovorus*.

Theo-F riboswitch was chromosomally knocked-in upstream of *Bd3318* directly after the TSS using the suicide plasmid pK18mobsacB.[37] After isolating the kanamycin resistant merodiploid, a second crossover event was induced by culturing in the presence of sucrose.

The Theo-F-*fliA* knock-in mutant was then compared to the wild-type for the plaque formation rate on double layer agar plates (Figure 3A). In the presence of theophylline (1 mM), the mutant plaques appeared at a similar rate as the wild-type (after 3 days). In the absence of theophylline, however, it took one additional day for the plaques to appear. Theophylline dependent predatory behavior was also observed in liquid culture. While the predation process of the Theo-F-*fliA* strain showed ~10 h delay compared to the wild-type, the delay was reduced to approximately 2 h in the presence of 1 mM theophylline (Figure 3B). Observation of a delay rather than complete suppression of predation may be due to low level of background FliA expression in the absence of theophylline. Further fine-tuning of the riboswitch characteristics (*e.g.*, lower OFF level) may lead to improved regulation of the predatory behavior.

Although *Bdellovibrios* were first discovered in 1963,[38] limited availability of genetic tools to manipulate gene expression remains a challenge for synthetic biology applications of these

DOI: 10.1021/acssynbio.7b00171
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bacteria with unique predatory life cycle. Some previous studies used heterologous promoters such as *E. coli lac* promoter and *nptII* promoter to drive expression of exogenous genes in *B. bacteriovorus*.[28,39] However, to take advantage of the biphasic life cycle of *B. bacteriovorus*, we explored the possibility of using strong native promoters that are specifically activated during the attack phase based on the global transcriptome data.[29] In this work, we evaluated four promoters that were suggested to be specifically active during the attack phase, and they all showed strong mCherry expression while *E. coli lac* promoter was marginally active (Figure 1). A recent study[40] showed that although *B. bacteriovorus* cells cannot replicate during this attack phase, they continue to uptake nutrients from the environment and use them to synthesize and secrete a wide range of proteins and hydrolytic enzymes. This is consistent with our observation that mCherry expression continues to increase even after 30 h when most of the prey cells were exhausted.

Our next step was to use some of these promoters to evaluate synthetic riboswitches that enable chemically inducible gene expression. While TPP riboswitches that were reported to work in *E. coli* did not function in *B. bacteriovorus*, some of the theophylline riboswitches from the Gallivan group were observed to be effective. It is notable that these riboswitches have been shown to function in a broad range of bacterial species.[24,26,27] In particular, we found that Theo-F exhibits respectable riboswitch properties in *B. bacteriovorus* with an ON/OFF ratio of approximately 40. Furthermore, the riboswitch was inserted into the chromosome to dynamically regulate expression of a gene that is critical for the predatory behavior.

As the field of synthetic biology advances toward more practical applications, it is likely that there will be increased needs to apply the technologies that have been developed in canonical host organisms such as *E. coli* to broader types of biological chassis.[41,42] The predatory bacterium *B. bacteriovorus* is a prime example whose unique biology offers promising applications as a chassis for synthetic biology. The results of the current study represent the first steps toward more sophisticated engineering of *B. bacteriovorus* and provide useful tools to study the basic biology of this interesting organism.

METHODS

Reagents. All PCR reactions were performed using Phusion High-Fidelity DNA Polymerase 2× Master Mix (New England Biolabs). Quick Ligation Kit (New England Biolabs) was used for DNA ligation reactions. Restriction enzymes were purchased from New England Biolabs. Theophylline, thiamine hydrochloride, TPP, and HEPES were purchased from Nacalai Tesque.

Microorganisms and Culturing Conditions. *B. bacteriovorus* HD100 strain was a kind gift from Dr. Robert J. Mitchell at Ulsan National Institute of Science and Technology, South Korea. This strain was kept as frozen glycerol stock. When needed, it was streaked on 1/10-diluted Nutrient Broth (DNB) double layer agar plates containing *E. coli* MG1655 as a prey in the upper layer.[43,44] The upper layer (10 mL) contained agar at 0.6% (w/v) while the lower layer (13 mL) contained agar at 1.2% (w/v). When appropriate, theophylline was added only to the upper layer at the required concentration. Liquid cultures of *B. bacteriovorus* were prepared using the same prey at an initial OD_{600} of approximately 1.0 in HEPES buffer (25 mM, pH 7.2). All *B. bacteriovorus* predatory cultures were supplemented with $CaCl_2$ (2 mM) and $MgSO_4$ (4 mM). The prey *E. coli* MG1655 was rendered kanamycin resistant by transformation with pEGFP-N1 plasmid (Clontech) harboring a kanamycin selection marker.

Plasmid Design, Construction, and Transformation. Plasmid vector pSUP404.2[28] was used for all experiments involving nonchromosomal mCherry expression. To maintain this plasmid in *B. bacteriovorus*, kanamycin (50 μg/mL) was added to liquid culture and to the upper layer for double layer agar plate experiments. For the promoter evaluation (Figure 1), the plasmids contained an additional GFPuv expression cassette under the native *B. bacteriovorus* promoter P_{2915} associated with *Bd2915* gene (Figure S3 and Table S1). This was intended to be used to normalize mCherry expression level, but the GFPuv expression level was observed to be insufficient for the purpose. Therefore, we opted to normalize mCherry fluorescence by *B. bacteriovorus* cell count determined by plaque assay, and the GFPuv expression cassette was not included in the riboswitch-containing plasmids (Figure S4). Plasmids used for promoter evaluation included ribosome binding site (RBS) sequences (for both GFPuv and mCherry) optimized for *B. bacteriovorus* HD100 using the RBS Calculator.[45] ThiM#2 and $ThiMN_{15}$#19 riboswitch sequences were PCR amplified from plasmids pLacthiM#2mCherry and pLacthiM19gfpuv, respectively.[32] The theophylline responsive riboswitches were constructed from synthetic oligonucleotides according to the published sequences.[24] Details of the plasmid sequences are provided in Figure S4 and Table S2. All the recombinant plasmids were constructed using *E. coli* TOP10 (Invitrogen) and sequence verified. The plasmids were then transformed into *E. coli* S17−1 and transferred to *B. bacteriovorus* through conjugation as described previously.[28]

Promoter and Riboswitch Evaluation. For the promoter evaluation experiments (Figure 1A, B), liquid predatory cultures were prepared for *B. bacteriovorus* harboring the appropriate plasmids. After 24 h, when all cultures were cleared, they were passed through 0.45 μm filters to remove *E. coli* cell debris. Ten microliters of the filtrate (containing approximately 2×10^6 PFU from each culture) were then added to 1 mL of fresh *E. coli* suspension in HEPES buffer supplemented with kanamycin (50 μg/mL) of which 200 μL were transferred to a 96-well plate. The plate was incubated at 37 °C with continuous shaking (270 rpm) in Tecan M1000PRO microplate reader while measuring OD_{600} and mCherry fluorescence (excitation: 587 nm, emission: 610 nm). Relative OD_{600} values normalized against the control wells without added predators are reported. For Figure 1C, the cultures were incubated at 30 °C within a shaking incubator (250 rpm) and samples were taken after 48 h for plaque counting and fluorescence measurement.

Riboswitch evaluations (Figure 2) were performed similarly with few exceptions. After the cultures cleared, Nutrient Broth was added to 0.1× final concentration (DNB). Aliquots (200 μL) of each culture were then transferred to a 96-well plate with and without an appropriate ligand. The cultures were then incubated at 30 °C for additional 48 h with continuous shaking (250 rpm) before fluorescence measurement and plaque assays were performed. It should be noted that although the recombinant pSUP404.2 plasmids were maintained in *B. bacteriovorus* HD100 by supplementing the culture medium with kanamycin (50 μg/mL), the number of kanamycin resistant cells tended to decrease to approximately 1/4 of the population after 48 h of incubation in DNB, possibly due to the extra metabolic load on the host bacterium.

DOI: 10.1021/acssynbio.7b00171
ACS Synth. Biol. 2017, 6, 2035−2041

Chromosomal Insertion of Theo-F Riboswitch. Chromosomal knock-in of the Theo-F sequence was accomplished using the suicide plasmid pK18mobsacB[37] as described previously.[46] Briefly, PCR was used to flank the Theo-F sequence with approximately 900 bp upstream of the *Bd3318* TSS and 900 bp downstream of the *Bd3318* start codon. This construct was then inserted into pK18mobsacB using *Xba*I and *Hin*dIII restriction sites. The recombinant plasmid was sequence verified and then transferred to *B. bacteriovorus* through conjugation. Initial selection of the merodiploid mutants was performed by spreading on double layer agar plates containing kanamycin (50 μg/mL) in the upper layer. After sequence confirmation, one merodiploid mutant plaque was cultured in predatory liquid culture without kanamycin to allow for the second crossover event. After the culture cleared, it was filtered through a 0.45 μm filter and liquid predatory subculture was made in the presence of sucrose at 2.5% (w/v) as well as theophylline (1 mM) to promote the second crossover event. After the clearance of the culture, mutants were screened on agar plates with theophylline (1 mM) added to the upper layer. The positive knock-in mutants were checked by PCR and sequenced to confirm the loss of the plasmid. Finally, the inability of the strain to grow in the presence of kanamycin was confirmed.

Predation Assays in Liquid Culture and on Agar Plates. The wild-type *B. bacteriovorus* and the Theo-F-*fliA* mutant strain were streaked on double layer agar plates and liquid predatory cultures were prepared with theophylline (1 mM). After clearance, the predatory cells were washed three times with HEPES buffer to remove theophylline. Liquid predatory subcultures were then made without theophylline to ensure removal of any traces of theophylline. These subcultures were then 0.45 μm filtered, and 10 μL of each culture (approximately 5×10^6 plaque forming units [PFU]) was added to 1 mL of *E. coli* suspension in HEPES buffer (adjusted to 1.0 OD_{600}) with and without theophylline (1 mM). Aliquots (200 μL each) were then transferred to a low evaporation 96-well plate (Nunc Edge 2.0 nontreated plates, Thermo Fisher Scientific) and incubated at 30 °C with continuous shaking (270 rpm) and OD_{600} measured in Tecan M1000PRO microplate reader. Relative OD_{600} values normalized against the control wells without added predators are reported. For the agar plate assay, the cultures were diluted and spread on double layer agar plates with or without theophylline (1 mM) added to the upper layer. The photos of the agar plates shown (Figure 3B) are representative of three independent cultures.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00171.

Supplementary Figures and Tables (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: yohei.yokobayashi@oist.jp.

ORCID

Yohei Yokobayashi: 0000-0002-2417-1934

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Dr. Robert J. Mitchell at Ulsan National Institute of Science and Technology in South Korea, Dr. Elizabeth Sockett at Nottingham University in UK and Dr. Eckhard Strauch at the Federal Institute for Risk Assessment in Germany for providing *B. bacteriovorus* HD100 strain, pK18mobsacB, and pSUP404.2, respectively. The research was funded by Okinawa Institute of Science and Technology Graduate University.

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