Protocol

1. Phage Isolation

- Plate 100 μL of normal *Escherichia coli* BL21 solution onto the LB plate.
- After drying, add 1 μL phage-infected fermentation solution at the center of the plate.
- Incubate the plate overnight at 37 °C.
- Separate the solid medium with the plaque from the plate, then put it into the solution for culturing the *E. coli* which has reached the logarithmic phase, culture it at 37℃ for 1-2h, preserve the supernatant in gel tube at -80 °C after the solution centrifugation.

2. Preparation And Crude Phage Particles

- 2.1 Prepare 100 mL of phage lysate, and then entrifuge the lysate at 10,000 rpm for 10 min, to obtain phage pure culture solution.
- 2.2 Prepare the crude phage particles: Firstly, inoculated 1 % *E. coli* overnight culture into 100 mL fresh LB medium, and cultured *Escherichia coli* to log phase (OD600≈0.4). Secondly, add 5 mL phage pure culture solution, and culture it in a shaking at 37°C and 180 rpm for 6 ~ 8 h. Then, add DNase I (5 μg/mL) and RNase A (5 μg/mL) to the lysate, and incubated 1 h at 37°C. Finally, add NaCl (0.1 M) to the solution, and mix them well and take ice bath for 1 h (or overnight).
- 2.3 After centrifuged the mixture at 12000 rpm for 20 min, transfer the supernatant to another centrifuge tube, and then add PEG6000 or (PEG8000) and ensure the final concentration is 10% (W/V). After the solution is fully dissolved, make it stand at 4℃ for 1 h.
- 2.4 Centrifuge the solution at 12000 rpm for 20 min and discard the supernatant. The pallet is then resuspended with 500 μL ddH2O, and use the same volume of chloroform to extract supernatant three times. Finally, centrifuge the solution at 12000 rpm for 10 min to remove PEG6000(PEG8000) from the suspension. And finally the phage particles is obtained.

3. Isolate The Phage DNA

- 3. 1 Add DNase I and RNase A to the purified phage particles to make it a final concentration of 1 μg/mL and keep it at 37 °C for 1 h to degrade the DNA or RNA of the remaining host bacteria.
- 3.2 Then add EDTA (pH 8.0) to a final concentration of 50 mmol/L to terminated the activities of DNase I and RNase A.
- 3.3 Add SDS to a final concentration of 0.5% (v/m), and proteinase K to a final concentration of 50 μg/mL, then keep it at 56℃ for 2 h to digest the protein.
- 3.4 Add an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1), and centrifuge at 10,000 rpm for 10 min to collect the supernatant.
- 3.5 After repeated step 4 for 3 times, the supernatant is collected, and equal volume of chloroform is added in, then centrifuge at 10000 rpm for 10 min, and the supernatant is collected;
- 3.6 After step 5, 1/10 volume of 3 mol/L NaAc and 2 volumes of ice 95% ethanol are added, and keep at -20 °C for at least 2 h. Then centrifuge at 10000 rpm for 10 min.

- 3.7 Add 70% ethanol (200 μL) to the pellet and invert the capped tube for several times and centrifuge at 12000 rpm for 5 min to recover the DNA of phage.
- 3.8 Discard the supernatant, and dry the open centrifuge tube for 10 min at room temperature, then resuspend the DNA with TE solution.
- 3.9 After dissolving completely, using 0.8% agarose electrophoresis to confirm whether phage DNA extracted successfully (mainly see if the strip is present, whether it is single).

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4. Determination of phage one-step growth curve

- 4.1 Culture 50 mL *E. coli* to log phase (OD600≈0.6).
- 4.2 Centrifuge at 4000 rpm for 10min to discard the supernatant.
- 4.3 Resuspend the pellet into 0.5 mL fresh liquid LB media, and then mix well with 0.5 mL phage lysate (keep MOI=0.001), and kept them at room temperature for 1 min.
- 4.4 After adsorption for 1 min, centrifuged at 13000 rpm for 30 S to remove free phage.
- 4.5 Resuspend the pellet into 100 mL fresh LB medium, start record the exact time and incubate at 37℃, 180 rpm.
- 4.6 At 3 min intervals, samples were taken.
- 4.7 Determine the infection centers of the phage by plaque assay.
- 4.8 Count the number of plaques(PFU) every time point, and analyse the result to derivative the latent period of phage.
- 4.9 Calculate the amount of released phage by dividing the titer of platform period phage by the titer of incubation period phage.

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5. Extraction of RNA from *Escherichia coli* Infected by Phage

- 5.1 Pellet the bacteria cells by centrifuging at 12000 rpm for 2 min at 4℃ and discard the supernatant.
- 5.2 Resuspend the pellet by 100 μL TE buffer with 400 μg/mL lysozyme, and incubated for it at room temperature for 3-5 min.
- 5.3 Add 350 μL Buffer RL (add 2-hydroxy-1-ethanethiol before use), mix thoroughly by vortex. If there is insoluble pellet present, centrifuge at 12000 rpm for 2min, and then transfer the clear supernatant to a new tube.
- 5.4 Add 250 μL absolute ethanol, and transfer all the solution and pellet to a new RNase-Free column CR3. Centrifuge at 12000 rpm for 30-60 s, discard the waste liquid, and put adsorption column back to collection tube.
- 5.5 Preparation of DNase I solution: add 10 mL DNase I stock solution to a new RNase-Free centrifuge tube, mix well with 70 μL Buffer RDD.
- 5.6 Add 80 μL DNase I solution into the central of RNase-Free column CR3, incubate 15 min at room temperature.
- 5.7 Add 350 μL Buffer RW1, centrifuge at 12000 rpm for 30-60 s, remove the supernatant, and put adsorption column back to collection tube.
- 5.8 Add 500 μL Buffer RW(add absolute ethanol before use), incubate for 2 min at room temperature, then centrifuge at 12000 rpm for 30-60 s, remove the supernatant, and put adsorption column back to collection column. Repeat this step for twice.
- 5.9 Centrifuge at 12000 rpm for 2min, discard the waste liquid, air dry the adsorption column for several minutes.
- 5.10 Transfer the adsorption column to a new RNase-Free centrifuge tube, and add 30-100 μL RNase-Free ddH2O exactly to the central of adsorption membrane. Incubate

for 2 min at room temperature, then centrifuge at 12000 rpm for 2min to obtain RNA solution.

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6. Selection of Inducible Promoters

- 6.1 Culture normal *Escherichia coli* and make it grow to OD600 about 0.6-0.8. Then select three samples labeled E0, E5 and E20. E0 means the *E. coli* is not infected by phages, E5 means *E. coli* infected by phages for 5 minutes, and E20 means *E. coli* infected by phages for 20 minutes. After centrifuged 50 mL of uninfected *Escherichia coli* solution at 4 °C, 8000 rpm for 20 min, the supernatant solution is discard and the pellet is rapidly frozen with liquid nitrogen to terminate the life activities of *Escherichia coli*. The bacteriophage that infected solution for 5min and 20 min are treated as described above.
- 6.2 Send three sets of samples to GENEWIZ company and obtain the transcriptome data. Based on transcriptome data, we select two inducible promoters. The first inducible promoter P_{putA} has extremely low gene expression level at 0 min of phage infection, but gene expression level significantly increases at 5 min. The second inducible promoter P_{glcF} has extremely low gene expression level at 0 min and 5 min of phage infection, but gene expression level significantly increases at 20 min. We used these two inducible promoters to respond to phage infection for 5 min and 20 min respectively.

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7. Characterisation of Inducible Promoters

- The inducible promoter P_{putA} and green fluorescent protein gene *gfp* are ligated at the compatible overhanging ends, and the P_{glcF} and red fluorescent protein gene *mCherry* are ligated. Then transform the ligations reactions into *E. coli* competent cells. After induction, the relative fluorescence intensity of different color fluorescent proteins is used to determine the strength of the inducible promoters.

7.1.Amplification of inducible promoters and fluorescent protein genes

7.1.1. PCR amplification system

Cloned Pfu DNA polymerase	25 μL
Primer 1	2 μL
Primer 2	2 μL
dd H20	20.5 μL
Template	0.5 μL

7.1.2. Recommended cycling parameters

98℃ 5：00
94℃ 0：30
55℃ 0：30 } ×30
72℃ 1：30
72℃ 1：00
4℃ ∞

7.1.3 Agarose Gel DNA Extraction Kit

Take 50 μL PCR products for agarose gel electrophoresis, and the DNA Marker with the maximum size of 2000bp is used as the control. Set the voltage 100 V and electrophoresis for 20 min. Cut the gel containing the target DNA under the ultraviolet lamp and put the gel into 1.5 mL centrifuge tubes.

Purify and recover the amplified genes with the gel recovery kit, and measure the gene concentration by taking 1 μL each.

7.2 PCR fusion

7.2.1 fusion system

gene 1	5 μL
gene 2	5 μL
Cloned Pfu DNA polymerase	10 μL

7.2.2 fusion conditions

98℃ 3：00
98℃ 0：08
61℃ 0：05 } ×13
72℃ 1：30
72℃ 10：00
4℃ ∞

7.2.3 amplification system

Template	4 μL
(this template is the amplification product of the previous PCR step)	
Primer 1	2 μL
Primer 2	2 μL
Cloned Pfu DNA polymerase	25 μL
ddH2O	17 μL

7.2.4 amplification conditions

98℃ 3：00
98℃ 0：10
56℃ 0：15 } ×30
72℃ 1：30
72℃ 20：00
4℃ ∞

7.2.5 Agarose Gel DNA Extraction Kit

Take 50 μL amplified products for agarose gel electrophoresis, and the DNA Marker with the maximum size of 2000bp is used as the control. Set the voltage 100 V and electrophoresis for 20 min. Cut the gel containing the target DNA under the ultraviolet lamp and put the gel into 1.5 mL centrifuge tubes.
Purify and recover the amplified genes with the gel recovery kit, and measure the gene concentration by taking 1 μL each.

7.3 Linearisation of circular plasmids

7.3.1 double enzyme digestion system

DNA	30μL
Enzyme 1	2.5 μL
Enzyme 2	2.5 μL (the use of enzymes depends on the design of primers, used here for the Hind III and BamH I)
10×Qcut	5 μL
ddH2O	10 μL

7.3.2 double enzyme digestion conditions

37℃ water bath insulation for 1 h.

7.4 Homologous recombination

7.4.1 homologous recombination system

5×CE II Buffer 4 μL
Exnase II enzyme 2 μL
Vector x μL
Gene y μL
x: y = Carrier base number×0.02/Carrier concentration: Gene base number ×0.04/Gene concentration

7.4.2 homologous recombination conditions

37℃ PCR instrument insulation for 30 min.

7.5 Transformation into *Escherichia coli* BL21

7.5.1 Add 10 μL plasmid into 1.5 mL centrifuge tube containing BL21 competent cells and ice bath for 45 min.
7.5.2 42℃ water bath the centrifuge tube for 45 S and put it back on ice immediately.
7.5.3 ice bath for 5 min.
7.5.4 Add 800 μL LB medium into the centrifuge tube and incubate it in the shaking table at 37℃ and 220 rpm for 60 min.
7.5.5 place the centrifuge tube in the centrifuge at 4000 rpm for 2 min.
7.5.6 discard the supernatant in the centrifuge tube, add 100 μL LB medium to re-suspend the bacteria, spread the re-suspended bacteria solution on the solid medium with corresponding resistance, and culture it upside down in the incubator at 37℃ for about 16 h.

7.6 Colony PCR

7.6.1 colony PCR system

2×Taq enzyme 10 μL
Primer 1 1 μL
Primer 2 1 μL
ddH2O 8 μL
Template
(select the bacterial colonies growing on the plate and blow and mix them in the PCR tube)

7.6.2 colony PCR conditions

98℃ 5: 00
94℃ 0: 30
55℃ 0: 30 } ×30
72℃ 1: 30
72℃ 1: 00
4℃ ∞

7.6.3 bacterial species preservation

Take 5 μL Products of colony PCR for agarose gel electrophoresis, and the DNA Marker with the maximum size of 2000bp is used as the control. Set the voltage 100 V and electrophoresis for 20 min. According to the electrophoresis results, the colonies which containing correct DNA fragments are selected and inoculated in 10 mL LB medium, and cultured in a shaking at 37 °C and 220 rpm overnight for about 16 h. Then 1 mL bacterial solution is taken and sequenced by GENEWIZ company. Take another 800 μL bacterial solution and 800 μL glycerol is mixed evenly and stored in the refrigerator at -40 °C.

7.7 Fluorescence Microscopy

7.7.1sampling

Inoculate 100 μL of preserved bacteria solution into 10 mL LB medium and place it in a shaking table at 37 °C and 220 rpm for overnight culture.
Inoculate 500 μL of overnight cultured bacteria solution into 50 mL LB medium and place it in a shaking table at 37 °C and 220 rpm for overnight culture.
When OD600 reach 0.6-0.8, take 1 mL of the sample as E0, then add 500 μL bacteriophage infection solution. After bacteriophage infection for 5 min, take 1 mL of the sample as E5. After bacteriophage infection for 20 minutes, take 1 mL of the sample as E20.
7.7.2 sample preparation
Centrifuge three groups of samples E0, E5 and E20 at 8000 rpm for 1 min.
Discard the supernatant and add 1 mL PBS to resuspend the bacteria.
Centrifuge at 8000 rpm for 1 min, discard the supernatant and repeat twice.
Finally, add 100 μL of PBS to the precipitated bacteria.
After the mixture, drop 1 μL on the slide and cover with the slide until it dried.

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8. Test of Anti-phage Protein AbpAB Searched from Papers

Clone the gene *abpAB* from the *Escherichia coli* BL21 genome and connect it to the pET28a plasmid containing the lactose operon. Use the final concentration is 0.5 mmol/L IPTG induce protein expression in chassis microorganisms *Escherichia coli* BL21.The induction temperature is 37 °C, And the induction time is 12 hours. Take samples at 0 h, 4 h, 6 h, 8 h and 10 h during induction and measure the values of OD600.
Compared with the growth curve of *Escherichia coli* with normal pET28a plasmid, the results show that the protein AbpAB have little effect on the growth of bacteria. Then, coat 100 μL of culture solution on the plate, and add 1 μL of phage solution to the center of the plate.

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9. Screening of Phage Resistant Mutant And Identification of Key Sites

The mutant of *Escherichia coli* BL21 is obtained by using the ARTP (Atmospheric and room temperature plasma) mutagenesis system.
Add bacteriophages into the mutant strain and place them in a shaking table at 37℃ and 180 rpm for 12 hours.
Coat the culture solution on the plate and incubate at 37℃ for 24 hours, the mutant strain that could grow a single colony on the phage is resistant to phage infection.
Inoculate all single colonies in 10 mL LB medium at 37℃ and incubate at 180 rpm for 12 h. Then coat 100 μL medium on the plate and add 1 μL phage solution to the center of the plate.
If plaque appear after overnight culture at 37℃, the mutant strain is eliminated. If no plaque appears, then mark the plate for culture, and select a single colony for culture and plate verification.
After 10 rounds of culture and plate verification, the mutant sites and bacteriophage resistance are consolidated, and 8 mutant strains are finally screened.
Sequence the eight mutant strains, and find the mutant genes shared by several mutant strains: *nuoE, yhjH, rzpD, gntR*.
Connect each of these genes to a pET28a plasmid with lactose operon. Use the final concentration is 0.5 mmol/L IPTG induce protein expression in chassis microorganisms

Escherichia coli BL21.The induction temperature is 37 °C, And the induction time is 12 h. Take samples at 0 h, 4 h, 6 h, 8 h and 10 h during induction and measure the values of OD600.
Compared with the growth curve of *Escherichia coli* with normal pET28a plasmid, and use the mathematical model the results show that *gntR* had the least effect on the growth of *Escherichia coli*.
After 12h of culture, coat 100 μL of culture solution to a plate with a final concentration of 0.5 mmol/L IPTG , and then add 1 μL of phage solution to the center of the plate and is cultured at 37℃ for 12 hours to observe the transparency of plaques, so as to determine which resistance gene has the best effect on phage resistance. The results show that *gntR* has the best effect on phage resistance.

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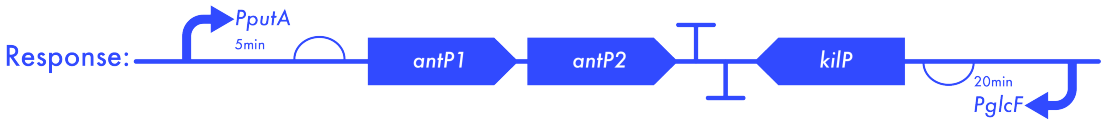
10. The Effectiveness of The Overall Gene Circuit

We ligated the combined resistance protein genes (*antP1-anpAB, antP2-gntR*) and the kill switch P-1 (BBa_K628000) after the latent phage-inducible promoter and the burst-inducible promoter, and performed the phage infection assay on the LB agar plate, and got very good resistance.



Others

- LB medium: NaCl 10 g/L, Yeast extract 5 g/L, Tryptone 10 g/L, 1 ml 1 N NaOH。
- LB plates: add 1.5% agar or agarose to LB medium
- Agarose gel electrophoresis
- Add 0.8% Regular agarose to TAE buffer. Add 0.02% fluorescent dye ethidium bromide (EB) after boiling water bath heating. Shake the solution well then fill it into a horizontal plastic frame and insert an appropriate size comb. After solidification, add DNA Marker to DNA samples and transfer them into the wells. Place the gel in the electrode buffer and electrophoresis under the condition that the electric field strength is not more than 5 V/cm. After the electrophoresis is finished, the DNA bands are observed under ultraviolet light, photographed by an ultraviolet analyzer or a gel imaging system, and relevant data analysis is performed.
- Petri dish preparation: Bath the solid medium in hot water until it is melted. When it is cooled to about 45 °C, add the required antibiotics, such as Kana (final concentration: 50 μg/mL) or Amp (final concentration: 100 μg/mL). Pour the melted gel into a clean culture dish, and pour 10-15 mL of the culture medium into each dish. (If no antibiotics are added, the medium can be directly poured into the culture dish.)
- Glycerol-preserving tube: add 800 μL of 50% glycerol to the bacteria-preserving tube. When the strain is preserved, add 800 μL of the bacteria solution. Mix the bacteria solution with glycerol to make the final glycerol concentration is 25%.