Attenuator LRR – a regulatory tool for modulating gene expression in Gram-positive bacteria

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Summary
With the rapid development of synthetic biology in recent years, particular attention has been paid to RNA devices, especially riboswitches, because of their significant and diverse regulatory roles in prokaryotic and eukaryotic cells. Due to the limited performance and context-dependence of riboswitches, only a few of them (such as theophylline, tetracycline and ciprofloxacin riboswitches) have been utilized as regulatory tools in biotechnology. In the present study, we demonstrated that a ribosome-dependent ribo-regulator, LRR, discovered in our previous work, exhibits an attractive regulatory performance. Specifically, it offers a 60-fold change in expression in the presence of retapamulin and a low level of leaky expression of about 1–2% without antibiotics. Moreover, LRR can be combined with different promoters and performs well in Bacillus thuringiensis, B. cereus, B. amyloliquefaciens, and B. subtilis. Additionally, LRR also functions in the Gram-negative bacterium Escherichia coli. Furthermore, we demonstrate its ability to control melanin metabolism in B. thuringiensis BMB171. Our results show that LRR can be applied to regulate gene expression, construct genetic circuits and tune metabolic pathways, and has great potential for many applications in synthetic biology.

Introduction
Over recent decades, the field of synthetic biology has undergone considerable growth. It aims to predict, create and control cellular behaviour in a rationally engineered way (Cameron et al., 2014; Green et al., 2014). Regulatory devices providing fine-tuned and optimized regulatory behaviour have been investigated within synthetic biology to regulate gene expression and construct synthetic networks (Groher et al., 2019). This has resulted in the development of protein-based inducible promoters (Lutz and Bujard, 1997), riboswitches (Serganov and Nudler, 2013; Boussebayle et al., 2019; Chi et al., 2019), and synthetic toehold switches (Green et al., 2014), as well as many other types of component.

Protein-based inducible promoters allow the regulation of gene expression by up to a thousand-fold range, such as the IPTG-induced lac promoter, the arabinose-activated ara promoter, and the tetracycline-induced tet promoter (Lutz and Bujard, 1997). They have been widely employed to tune conditional protein expression in E. coli. However, inducible promoters are often subject to high rates of leaky expression, require transcription factors to function well (Hansen et al., 1998; Giacalone et al., 2006; Zhang et al., 2012; Rosano and Ceccarelli, 2014) and can cause metabolic burden upon the host cell because of the cost of translation of the transcription factors (Keny and Dixon, 2019). Additionally, limitations have been observed when these regulatory elements are employed in diverse hosts (Fukui et al., 2011). For example, protein-based regulatory tools derived from E. coli have been often found to not function well in other bacteria, such as B. subtilis (Cui et al., 2016).

To avoid the limitations of protein-based regulatory tools, RNA-based regulatory tools have been increasingly viewed as excellent candidates to tune and control gene expression in synthetic biology (Ma et al., 2014). Non-coding RNAs are essential tools to regulate cellular processes. They have distinct advantages over protein-based systems, such as fast regulatory response, genetic modularity, portability and the possibility of being combined with other platforms (Chappell et al., 2017; Boussebayle et al., 2019). Among these tools, riboswitches and RNA regulators that use base pairing (such as small transcription activating RNAs (STARs), toehold repressors, three-way junction (3WJ) repressors, and toehold switches) are believed to hold potential for
application in synthetic biology (Chappell et al., 2017; Kim et al., 2019; Chau et al., 2020).

Riboswitches are newly discovered cis-modulatory elements controlling various basic metabolisms of living cells (McCown et al., 2017). They consist of two connected RNA parts (Suess et al., 2004), an aptamer domain that senses and binds a target ligand with high affinity and specificity, and an expression platform that undergoes a conformational change to control gene expression after ligand binding (Goodson et al., 2017; Shanidze et al., 2020). Riboswitches recognize a wide array of small metabolites, antibiotics and ions in cells, and directly tune gene expression based on the concentration of the specific ligand. They utilize various mechanisms to modulate target gene expression, such as sequestering or freeing the ribosome binding site (RBS), attenuating RNA transcription, splicing regulation and influencing RNA stability (Serganov and Nudler, 2013; Breaker, 2018; Pavlova et al., 2019). These attractive advantages have resulted in riboswitches showing great promise as regulatory tools and are being widely explored and developed for applications in synthetic biology (Chi et al., 2019). However, their limited dynamic range and context-sensitive performance often restrict their utility (Dwidar and Yokobayashi, 2019; Kent and Dixon, 2019). Additionally, the application of riboswitches in bacteria with different genetic contexts raises the problem of conformational stability (Chen et al., 2012). Moreover, the cognate ligands of natural riboswitches are small metabolites and metal ions, which might be competitively bound by other factors. As a result, the expression of the target gene might not be tuned by its ligand in a predicted dose-dependent manner (Nshogozabahizi et al., 2019). Thus, a small fraction of riboswitches is suitable for application and most synthetic riboswitches have only been used for proof-of-concept studies (Nshogozabahizi et al., 2019). The bottleneck of synthetic riboswitch development may be the screening of fit-for-purpose aptamers possessing both excellent binding properties and conformational flexibility (Groher et al., 2018).

In contrast, RNA regulators modulating gene expression through Watson-Crick base pairing can provide a more than 100-fold change in gene expression upon activation, for example, functioning as toehold switches (Callura et al., 2010; Green et al., 2014; Green et al., 2017), toehold repressors, 3WJ repressors (Kim et al., 2019), STARs (Chappell et al., 2017; Bartoli et al., 2020a), RNA transcription–translation regulators (Westbrook and Lucks, 2017), and tunable expression systems (Bartoli et al., 2020b) plus many more.

All these RNA regulators comprise two separate RNA parts driven by different promoters. One RNA sequence in the 5′-untranslated region of the target gene controls gene transcription or translation by forming a terminator or sequestering the RBS, while the other plays a role in activating or repressing the expression of the target gene by changing the structure of the first RNA through base pairing. Toehold switches, toehold repressors and 3WJ repressors consist of a designed switch RNA and trigger RNA. The switch RNA lies upstream of the target gene. When the trigger RNA binds to the switch RNA, the latter will refold and change the accessible state of the RBS, thus regulating gene expression. These tools tune gene expression only at the translation level (Callura et al., 2010; Green et al., 2014; Kim et al., 2019). Additionally, STARs, a powerful class of RNA-based transcriptional activators, comprise a target RNA located upstream of the gene of interest and a STAR (Chappell et al., 2017). In the OFF state, the target RNA folds into an intrinsic terminator structure that inhibits transcription of the downstream gene, whereas in the ON state, a STAR is transcribed that interacts with the target RNA through base pairing, preventing terminator hairpin formation and allowing transcription of the downstream gene (Chappell et al., 2017). Excitingly, RNA regulators based on base pairing can be designed computationally allowing for large libraries to be constructed and offer high-performance and orthogonal gene expression regulation in diverse contexts (Chappell et al., 2017), which is difficult for riboswitches. However, one drawback of RNA regulators is that their two RNA parts are driven by different promoters, which enlarges and complicates the genetic circuits.

RNA-based attenuators have been known about for several decades. An attenuator is a cis-regulatory RNA element located in the 5′-untranslated region of its downstream gene, and controls gene expression by sensing antibiotics and amino acid starvation (Konan and Yanofsky, 1997; Dar et al., 2016). In the presence of antibiotics and amino acid starvation conditions, the RNA structure of the attenuator changes upon transient ribosome stalling, which forms an anti-terminator or anti-sequester structure to generate a high expression level of the downstream gene. Most inducers of attenuators are antibiotics like lincomycin (Dar et al., 2016; Duval et al., 2018), tetracycline (Wang et al., 2005), chloramphenicol (Bruckner and Matzura, 1985), erythromycin (Duval et al., 2018) and so on. Additionally, some attenuators are induced upon amino acid starvation, such as a lack of tryptophan (Konan and Yanofsky, 1997). There has been little reported research on the application of attenuators.

In this study, we show that the attenuator, LRR, reported in our previous publication, offers a satisfactory fold change over 60-fold and a low rate of leakage expression of less than 2%. Moreover, LRR exhibited a fine regulatory performance when combined with
different promoters and successfully controlled melanin metabolism in its natural host *B. thuringiensis* BMB171. LRR can also play a switching role in *B. cereus*, *B. amyloliquifaciens*, *B. subtilis*, and *E. coli*. Therefore, we believe that attenuator LRR has potential as a candidate tool for synthetic biology.

**Results**

*A natural ribosome-dependent attenuator with excellent regulatory behaviour*

In our previous study, we identified a ribosome-dependent attenuator, termed LRR, which regulates the rppA gene (encoding a ribosomal protection protein) expression in *B. thuringiensis* BMB171 (Cai et al., 2020). Attenuator LRR consists of a simple RNA part and modulates gene expression through conformational change resulted from transient ribosome stalling. In the presence of antibiotics chloramphenicol (Cam) or virginiamycin (Vgm), LRR is in the ‘on’ state and leads to increased expression of the downstream gene (Fig. 1A; Cai et al., 2020). To gain a deeper understanding of the characteristics of LRR, other ribosome-targeting antibiotics were tested to induce the ‘on’ state of LRR. The results demonstrated that antibiotics retapamulin (Rtm), Vgm, Cam, pristinamycin IA (Pnm) and linezolid (Lnz) markedly induced the ‘on’ state of LRR (Fig. 1B). Among them, Rtm caused an increase in expression of over 60-fold. Regulatory performances of certain natural and synthetic riboswitches commonly used in synthetic biology were analysed and recorded in Table 1. The performance of LRR induced by Rtm is superior to most reported natural and synthetic riboswitches. Therefore, LRR is considered as a promising potential tool for application in synthetic biology. Next, we explored its regulatory performance when driven by Rtm.

**Characterization of the dose dependence and activation ratio of LRR**

To examine whether the switching activity of LRR is dependent on the dose of antibiotic Rtm, we used the previously engineered strain BMB171/pB-PLR, which carries the rppA promoter and LRR fused to the lacZ gene (Cai et al., 2020). The strain was cultured in 20 ml of Luria-Bertani (LB) medium supplemented with different concentrations of Rtm for 6 h. Then, the β-galactosidase activities were measured to determine the dose dependency. The LacZ expression level was enhanced as the concentration of Rtm increased from 0 to 0.7 µg ml⁻¹ and then decreased gradually when the Rtm concentration increased from 0.8 to 2 µg ml⁻¹ (Fig. 2A). These results suggested that the appropriate dose of Rtm induced a higher expression level of LacZ.

To check for non-specific effects of Rtm, the β-galactosidase activities of strain BMB171/pB-P treated with different concentrations of Rtm (0, 0.5, and 0.7 µg ml⁻¹) were measured. Plasmid pB-P harbours the same promoter PrppA and RBS as pB-PLR, but has no LRR. The results demonstrated that Rtm did not significantly affect the activity of promoter PrppA (Data S1). Therefore, the higher expression of LacZ in strain BMB171/pB-PLR induced by Rtm resulted from the presence of attenuator LRR. Interestingly, a low concentration of Rtm promoted the growth of bacteria rather than inhibited it (Fig. 2C). This might be because of the stimulatory effect of a low dose of toxic substances, termed hormesis (Calabrese and Baldwin, 2003).

The fold change in expression caused by 0.5 µg ml⁻¹ Rtm was about 63.9-fold, while that of 0.7 µg ml⁻¹ Rtm was only about 66.5-fold. Therefore, for reasons of economy, Rtm at 0.5 µg ml⁻¹ was selected to activate LRR. Then, the activation ratio elicited by Rtm was investigated. Strain BMB171/pB-PLR was incubated in 20 ml of LB medium supplemented with 0.5 µg ml⁻¹ Rtm for 16 h, and the LacZ expression and growth curve of the bacteria were quantified at the indicated time points. The result indicated that the lacZ expression level increased gradually during the induction period (Fig. 2B). After 16 h of induction, the activation ratio was about 64.4. Activation ratio is calculated by dividing the Miller units (β-galactosidase activity) in the presence of Rtm by the Miller units in the absence of Rtm. The strain BMB171/pB-PLR grew similarly in LB medium with or without 0.5 µg ml⁻¹ Rtm (Fig. 2D).

**Characterization of the leaky expression of LRR**

In this study, leaky expression was evaluated by assessing the expression of a lacZ reporter gene driven by the ‘off’ state of LRR without antibiotics. Leaky expression was calculated by dividing the Miller units (β-galactosidase activity) in the presence of LRR by the Miller units in the absence of LRR. The result showed that the leaky expression level was about 0.8% when LRR was driven by its natural promoter PrppA derived from the rppA gene (Fig. 3A). Furthermore, the leaky expression of LRR driven by other promoters was also explored. The PrppA promoter was replaced with other promoters (P_{rppA}, PyliA, and PyhfH) in plasmids pB-P and pB-PLR to generate plasmids pB-P_{rppA}, pB-P_{PyliA}, pB-P_{PyhfH}, pB-P_{PyhfH-LR}, and pB-P_{PyhfH-LR}, respectively. P_{rppA} is the mutant of promoter PrppA with a higher activity than PrppA. PyhfH was derived from gene BMB171_RS05540 with lower activity than PrppA, while PyliA was derived from gene yifA encoding a sigma (54) modulation protein, and its activity is similar to PrppA. Results showed that the leaky expression of LRR driven

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Regulatory behaviour of LRR driven by different promoters

For an inducible expression system to be broadly useful, it is important that it functions well when used in combination with other genetic elements (Boussebayle et al., 2019). Subsequently, regulatory behaviour of LRR driven by other promoters was investigated. The fold change in expression induced when LRR was driven by promoters $P^M$, $P_{yifA}$, and $P_{yhfH}$ was 44.3, 26.8, and 34.8, respectively (Fig. 3B). The data indicated that LRR has satisfactory regulatory behaviour when driven by other promoters. This compatibility characteristic represents another advantage of LRR when used to modulate diverse gene expression.

Investigating regulatory behaviour of LRR in other bacteria

For the LRR attenuator to be broadly applicable across synthetic biology, it is also important that it is able to function in other types of bacteria. Thus, we investigated regulatory behaviour of LRR in $B. subtilis$, $B. cereus$, and $B. amyloliquefaciens$. The results indicated that LRR driven by the $P_{rppA}$ promoter has satisfactory regulatory behaviour in $B. subtilis$, $B. cereus$, and $B. amyloliquefaciens$, displaying a fold change in expression of 30, 93.6, and 34, respectively (Fig. 4A). We also investigated regulatory behaviour of LRR when driven by a 

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Table 1. Regulatory performance of certain reported riboswitches.

| Riboswitches                   | Organism          | Inducer concentration | State (+/-I) | Fold-change | Synthetic/natural | References               |
|--------------------------------|-------------------|-----------------------|--------------|-------------|-------------------|--------------------------|
| Caprolactam riboswitch         | E. coli           | 50 mM                 | ON           | 3.36        | Synthetic         | Jang et al. (2019)       |
| Ciprofloxacin riboswitch       | Yeast, human      | 1 mM                  | OFF          | 7.5         | Synthetic         | Groher et al. (2018)    |
| Neomycin riboswitches          | Saccharomyces     | 100 μM                | OFF          | 7.5         | Synthetic         | Weigand et al. (2008)   |
|                                | cerevisiae        |                       |              |             |                   |                          |
| Paromomycin riboswitch         | S. cerevisiae     | 250 μM                | OFF          | 8.5         | Synthetic         | Boussebaye et al. (2019) |
| Theophylline riboswitch        | B. subtilis       | 8 mM                  | ON           | 6.8         | Synthetic         | Cui et al. (2016)       |
| Tetracycline riboswitch        | S. cerevisiae     | 250 μM                | OFF          | 40          | Synthetic         | Groher et al. (2019)    |
| Theophylline riboswitch        | E. coli           | 500 μM                | OFF          | 6           | Synthetic         | Suess et al. (2003)     |
| Theophylline riboswitch 8.1    | E. coli           | 1 mM                  | ON           | 36          | Synthetic         | Desai and Gallivan (2004)|
| Theophylline riboswitch 8.1*   | E. coli           | 1 mM                  | ON           | 59          | Synthetic         | Lynch and Gallivan (2009)|
| Theophylline riboswitch 12.1   | E. coli           | 1 mM                  | OFF          | 27          | Synthetic         | Lynch and Gallivan (2009)|
| Theophylline riboswitch        | E. coli           | 1 mM                  | OFF          | 36          | Synthetic         | Lynch and Gallivan (2009)|
| Pyrimido [4,5-d] pyrimidine-2,4-diamine riboswitch | E. coli | 1 mM | ON | 80 | Synthetic | Kent and Dixon (2019) |
| Histamine riboswitch           | Artificial cells  | 5 mM                  | ON           | 30.7        | Synthetic         | Dwidar et al. (2019)    |
| Mg²⁺ riboswitch                | Salmonella enterica | 25 μM                | OFF          | 25          | Natural           | Cromie et al. (2006)   |
| Glycine riboswitch             | Clostridium        | 0.1 mM                | OFF          | 10.2        | Natural           | Zhou et al. (2019)      |
|                                | pasteurianum      |                       |              |             |                   |                          |
| Lysine riboswitch              | E. coli           | 1 mM                  | OFF          | – 10        | Natural           | Zhou and Zeng (2015)    |
| pubE riboswitch (2-aminopurine-responsive) | Geobacillus | 2 mM | ON | – 29 | Synthetic | Marcano-Velazquez et al. (2019)|
| thermoglucosidasium            |                   |                       |              |             |                   |                          |
| N-acetylneuraminic acid riboswitch | E. coli | 12.9 mM | OFF | 5.8 | Synthetic | Pang et al. (2020) |
| Fluoride-responsive riboswitch | Pseudomonas putida | 15 mM                | ON           | 3.8         | Natural           | Calero et al. (2020)    |

constitutive promoter, P43, derived from B. subtilis. The changes in expression induced by LRR were 15 and 6.6-fold in B. amyloliquefaciens and B. subtilis, respectively. Therefore, LRR could be used to tune gene expression and control metabolic pathways in B. subtilis, B. cereus, and B. amyloliquefaciens.

Additionally, we observed regulatory behaviour of LRR in the Gram-negative bacterium E. coli, using rfp as the reporter gene. The LRR coding region and rfp were inserted into plasmid pET-28(a) to generate the plasmid pET-LRR-RFP, and the T7 promoter was used to drive gene expression. The E. coli strain BL21 harbouring pET-LRR-RFP was cultured with and without antibiotics Rtm, Cam, Pnm, Tet, and Vgm at 37°C for 10 h, and then 2 ml of these cultures were collected and suspended in phosphate-buffered saline (PBS). The fluorescence intensity of these samples was detected and normalized to the cell density. The results demonstrated that LRR driven by the T7 promoter could regulate gene expression in E. coli. With the addition of Rtm, the expression of RFP increased, and the expression of RFP increased by approximately ninefold (Fig. 4B).

Tight regulation of melanin metabolism using LRR

In B. thuringiensis BMB171, melanin metabolism involves three enzymes encoded by an operon containing the hppD, fahA, and hmgA genes (Fig. 5A). The product of hppD is 4-hydroxyphenylpyruvate dioxygenase (HppD), which catalyses the synthesis of homogentisate from 4-hydroxyphenylpyruvate. Homogentisate molecules are changed to melanin via spontaneous oxidation and polymerization (Yang et al., 2018). The hmgA gene encodes homogentisate-1,2-dioxygenase (HmgA) that degrades homogentisate to 4-maleylacetoacetate, which is isomerized by maleylacetoacetate isomerase (MaIA) to fumarylacetoacetate. The fahA gene encodes fumarylacetoacetate hydrolase, which hydrolyses fumarylacetocetate to fumarate and acetoacetate (Fig. 5B; Tan et al., 2019). HppD is the key enzyme for melanin synthesis, while HmgA and FahA play a role in decreasing melanin production by degrading homogentisate. To test its effectiveness and compatibility to regulate bacterial metabolism, LRR was used to control melanin metabolism by tuning hppD expression.
To assess the potential for LRR-based control of melanin metabolism, a high copy number plasmid pB-hppD, carrying LRR and hppD, was constructed and transformed into strain DHFA (Fig. 6A). Homogentisate molecules are not synthesized and degraded in strain DHFA because the genes hppD, fahA and hmgA have been deleted. Therefore, the production of melanin in strain DHFA/pB-hppD would be caused by the hppD gene in plasmid pB-hppD.

Strain DHFA/pB-hppD was cultured in LB medium (with 1% Tyr) supplemented with or without Rtm (0.5 μg ml⁻¹, 0.965 μM). The lacZ expression level was measured by detecting β-galactosidase activity and calculated from three biologically independent replicates and shown as the mean ± SD. Cell density was evaluated using optical density at 600 nm (OD600). The final concentrations of Rtm were shown as bellow: 0, 0.3 (0.579), 0.4 (0.772), 0.5 (0.965), 0.6 (1.158), 0.7 (1.352), 0.8 (1.545), 0.9 (1.738), 1 (1.931), 1.25 (2.414), 1.5 (2.897), 1.75 (3.38) or 2 (3.863) μg ml⁻¹ (μM).

Strain DHFA/pB-hppD cultures turned dark brown, indicating that melanin had accumulated successfully in strain DHFA/pB-hppD. In contrast, the control group and the DHFA/pB-hppD strain without Rtm treatment cultured for the same time exhibited no melanin production. The optical densities at 400 nm (OD400) of all strains at different time points are shown in Fig. 6B, and those of the cultures grown for 60 h are shown in Fig. 6D. Additionally, the inducible fold change in expression of the hppD gene at different time points was calculated using quantitative real-time reverse transcription PCR (qRT-PCR) data and shown in Fig. 6C. The expression of HppD

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protein was also detected using Western blotting with an anti-Flag antibody (Fig. 6E). These results demonstrated that LRR successfully controlled melanin metabolism by regulating the expression of the *hppD* gene.

**Discussion**

In the present study, we characterized a natural attenuator, LRR, as a promising genetic switch to control melanin metabolism. LRR displays > 60-fold change in expression (in the presence of Rtm) and a low rate of leaky expression (without Rtm). In addition, the compatible characteristics of LRR enable it to function well when driven by different promoters. Although LRR is derived from *B. thuringiensis*, it also exhibited good switching behaviour in *B. subtilis*, *B. cereus*, *B. amyloliquefaciens*, and *E. coli*. Therefore, LRR is a potentially useful regulatory tool for synthetic biology applications.
LRR-based regulation relies on a change in RNA structure. Similar to riboswitches, RNA elements that modulate gene expression by changing their spatial RNA structure. Attenuators and riboswitches are both cis-regulatory RNA elements that modulate gene expression by changing its spatial RNA structure. Similar to riboswitches, LRR-based regulation relies on a change in RNA conformation caused by transient ribosome stalling. This characteristic facilitates the manipulation and application of LRR in synthetic biology. However, compared with protein-basing regulators, synthetic theophylline riboswitches with a robust activation ratio (96-fold) (Table 1), and RNA regulators based on base pairing, the performance of the LRR attenuator has room for improvement. Fortunately, the background level of target gene expression driven by LRR is low; therefore, LRR is more suitable to control the expression of gene encoding proteins that have detrimental or repressive effects on bacteria. The directed evolution of a version of LRR with a higher activation ratio could be sought in the future to reduce the gap with other regulatory parts.

A riboswitch recognizes its ligand with high affinity and specificity, and its aptamer usually senses one metabolite or metal ion. Only a few riboswitches can recognize two ligands, such as the Ni\(^{2+}\)/Co\(^{2+}\) responsive riboswitch (Furukawa et al., 2015). Compared with riboswitches, attenuator LRR is activated by several ribosome-targeting antibiotics, for example Vgm, Cam, Lnz, Pnm and Rtm. This behaviour may be an advantage, allowing multiple inputs to drive expression of a target gene in a dose-dependent manner. By comparison, small metabolites and metal ions sensed by riboswitches might not perform so predictably as they might bind competitively with other factors in bacteria.

Attenuators have been known about for several decades, and most of the previous studies focused on their working mechanism. In the presence of ribosome-targeting antibiotics or amino acid starvation, attenuators change their RNA structure through transient ribosome stalling and regulate downstream gene expression. This feature suggests that attenuators might only be suitable for bacteria because there is no spatial coupling between transcription and translation in eukaryotic cells (Gowrishankar and Harinarayanan, 2004; McGary and Nudler, 2013; Zhu et al., 2019). According to our results, LRR could play a regulatory role in Gram-positive bacteria, such as B. thuringiensis, B. subtilis, B. cereus, B. amyloliquefaciens, as well as in Gram-negative bacterium E. coli. More bacteria should be tested in the future to determine if LRR maintains its regulatory role.

Attenuator LRR regulates the expression of an antibiotic resistance-associated protein RppA (Cai et al., 2020); however, RppA does not seem to help bacteria improve their resistance to Rtm. However, unlike previously reported attenuators, LRR can promote significant increases in downstream gene expression. More importantly, the concentration of Rtm that can effectively induce LRR to refold has no significant detrimental effect on the host cells. Therefore, we believe that LRR has the potential to be applied as a regulatory tool. As the field of synthetic biology grows dramatically, the demand
for regulatory tools is increasing. The discovery and application of attenuator LRR will enrich the variety of available switching tools and stimulate the discovery of other kinds of regulatory tools.

**Experimental procedures**

*Primers, plasmids, bacterial strains and culture conditions*

The primers, plasmids and bacterial strains used in this study are listed in Tables S1–S3 respectively. Primers were synthesized by GENEWIZ (Beijing, China). The *E. coli* strains were cultured in LB medium at 37°C with shaking at 200 r.p.m. *B. thuringiensis, B. cereus, B. subtilis* and *B. amyloliquefaciens* were incubated in LB medium at 28°C with shaking at 200 r.p.m. Ampicillin (GenStar, Beijing, China) (100 μg ml⁻¹) for *E. coli* and erythromycin (GenStar) (50 μg ml⁻¹) for *B. thuringiensis, B. cereus, B. subtilis* and *B. amyloliquefaciens* were supplemented as required.

*Antibiotic-induction experiments*

For the antibiotic-induction experiments, strains carrying certain plasmids were grown overnight in LB medium at 28°C with shaking at 200 r.p.m. Overnight cultures were diluted (OD₆₀₀ = 0.2) in LB medium. Antibiotics Rtm, tetracycline (Tet), Vgm, rapamycin (Rap), lincomycin (Lin), Pnm, Cam, Lnz, telithromycin (Tel), kanamycin (Kan) and vancomycin (Van) (MedChem Express, Monmouth Junction, NJ, USA) were added as required and cultivation was continued for different times. Then, 2 ml of the cultures was harvested to assay the β-galactosidase activity or to extract the total RNA.

*Plasmid construction*

All constructs used in this study were based on the plasmids pHT1K or pET-28(a). The target DNA fragments were generated by PCR using the primers listed in Table S1. Then, DNA fragments containing the LRR

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![Pathway for the synthesis of homogentisate (Yang et al., 2018).](image-url)

A. Genes *hppD*, *fahA*, and *hmgA* in BMB171 constitute an operon and are drawn using light blue arrow. Other genes are indicated using grey arrow.

B. Homogentisate pathway for the catabolism of tyrosine. Enzyme names for each step are indicated above the black arrows.
coding region and promoters PM, PyhfH, PyifA or P43 were ligated into plasmid pHT1K via the SalI-NcoI sites to generate plasmids pB-PMR, pB-PyhfH-LR, pB-PyifA-LR and pB-P43-LR. Plasmid pB-PLR was constructed in a previous study (Cai et al., 2020). Compared with plasmids pB-PMR, pB-PyhfH-LR, pB-PyifA-LR and pB-pLR, the LRR encoding region was deleted in plasmids pB-PM, pB-PyhfH, pB-PyifA and pB-p, and their lacZ gene shares the rppA RBS. The sequences of the LRR coding region and all promoters used in this study are listed in the supplementary material.

Plasmid pB-hppD was generated by replacing the lacZ gene in plasmid pB-PLR with the hppD gene. A Flag-tag coding sequence was ligated to the 3’ end of the hppD gene, which encoded a Flag-tagged HppD protein that could be detecting using Western blotting. Plasmid pET-LRR-RFP was constructed by inserting the LRR coding region and the RFP open reading frame into plasmid pET-28(a) via the XbaI-XhoI sites. In plasmid pET-LRR-RFP, LRR and RFP expression were driven by a T7 promoter. All plasmids were constructed according to the manufacturer’s instructions using the pEASY®-Basic Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China), and all ligation reactions were transformed into E. coli DH5α competent cells. After confirmation by sequencing (GENEWIZ), recombinant plasmids were extracted from DH5α cells and transformed into the indicated competent cells via electroporation for further experiments.

Fig. 6. Adopting LRR for tight regulation of melanin metabolism.
A. Schematic presentation of LRR application for controlling melanin synthesis. Gene hppD was fused to LRR and rppA promoter in high copy number plasmid pB-hppD. In the presence of Rtm, the strains harbouring plasmid pB-hppD produce melanin. Reversely, the melanin production is inhibited in the absence of Rtm.
B. The OD400 value of different strains in LB medium supplemented with or without 0.5 μg ml⁻¹ Rtm. Strain ΔHFA was used as control.
C. Change folds of the hppD expression level induced by 0.5 μg ml⁻¹ Rtm at different time points were measured using qRT-PCR. Change folds were obtained by dividing the relative expression level of the hppD gene in the presence of Rtm by the data in the absence of Rtm.
D. The culture supernatant of different strains with or without 0.5 μg ml⁻¹ Rtm.
E. Western blotting of HppD fused with a Flag tag in different strains. Protein EF-Tu was used as a loading control.
β-galactosidase activity assay

The expression level of the LacZ protein in various strains was measured by determining β-galactosidase activity. The β-galactosidase activity was determined according to a previously described method and normalized to the cell density (Cai et al., 2020). Two millilitres of bacterial cultures was subjected to centrifugation to collect the cells, which were suspended in 1 ml of Z-buffer (0.06 M Na₂HPO₄, 0.04 M NaH₂PO₄, 0.01 M KCl, 0.001 M MgSO₄, 0.05 M β-mercaptoethanol, pH 7.0). Then, cell disruption was performed using a Tissue Cell-destroyer 1000 (Hubei Xinzongke viral Disease Control Bio-Tech, Hubei, China). The treated sample (200 μl) was added with 800 μl of Z-buffer and then incubated at 37°C for 5 min. The reaction was activated by the addition of 200 μl of ortho-Nitrophenyl-β-galactoside (ONPG) at 37°C for 1–10 min, followed by adding 500 μl of 1 M Na₂CO₃ solution to stop the reaction. The reaction times were recorded. The absorbance of the reactions at 420 nm (OD₄₂₀) was measured using an EnSpire™ Multimode Plate Reader (Perkin-Elmer, Waltham, MA, USA). Finally, the OD₄₂₀ value was converted into Miller units.

Fluorescence intensity assay

The expression of RFP for strains treated with or without antibiotics was measured by determining the fluorescence intensity. Cultures grown at 37°C with shaking at 200 r.p.m. for 10 h were centrifuged to assay fluorescence intensity. The collected bacterial cells from 2 ml cultures were washed with PBS three times and resuspended in 1 ml of PBS. The fluorescence intensity of these suspensions was assayed in an EnSpire™ Multimode Plate Reader (Perkin-Elmer) using an excitation wavelength at 584 nm and an emission wavelength at 607 nm. The cell density (OD₆₀₀) of samples was also recorded. The expression level of RFP was represented by the relative fluorescence units (FI/OD) calculated by dividing the fluorescence intensity (FI) by the cell density (OD).

Measurement of melanin production

To monitor melanin production, strains were incubated overnight in LB medium. The overnight cultures were diluted (OD₆₀₀ = 0.2) in 20 ml of LB medium in a 250 ml flask and grown at 28°C with shaking at 200 r.p.m. Rtm was added to a final concentration of 0.5 μg ml⁻¹. Then, 2 ml of cultures grown for 12, 24, 36, 48 and 60 h was centrifuged and melanin production was determined by measuring the optical density at 400 nm (OD₄₀₀) using the EnSpire™ Multimode Plate Reader (Yang et al., 2018).

Construction of the growth curve

The growth curve of strains was evaluated using their optical density at 600 nm (OD₆₀₀). The bacteria were cultured overnight in LB medium. The overnight cultures were diluted (OD₆₀₀ = 0.2) in 20 ml of LB medium (in a 50 ml flask) supplemented with or without certain antibiotics, and incubated at 28°C with shaking at 200 r.p.m. Then, the optical density of the cultures was measured at different time points to construct the growth curve.

RNA extraction and qRT-PCR

Total RNA was extracted using the TRIzol reagent (Invitrogen, Waltham, MA, USA) and then quantified using NanoDrop technology (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Shiga, Japan) according to the manufacturer's instructions. The cDNA was then used as the template for quantitative real-time PCR (qPCR). The 16S rRNA gene was used as an internal control (Cai et al., 2020).

Western blotting

Isolation of total bacterial proteins and determination of protein levels by Western blotting following SDS-PAGE were performed according to a previously described method (Jiang et al., 2018). The primary rabbit monoclonal antibody recognized the Flag-tag (DYKDDDDK Tag (D6W5B)) (Cell Signaling Technology, Danvers, MA, USA) and the secondary antibody, horseradish peroxidase (HRP)-conjugated Goat Anti-Rabbit IgG (H + L) (Abclonal, Wuhan, China), were used to probe the Flag-HppD protein. EF-Tu served as a loading control (Duaal et al., 2018) and was probed using an anti-eEF1A1/EF-Tu primary antibody (Abcam, Cambridge, MA, USA) and an HRP-Goat Anti-Mouse IgG (H + L) secondary antibody (Abclonal).

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J.C. and X.C. designed the study. X.C. carried out most of the experimental work and analysed the data. Q.W. and D.Y. constructed several plasmids. Y.F., B.A. and Y.Z. constructed the strain ΔHFA. X.C. wrote the manuscript. J.C., B.Y. and X.C. revised the manuscript. This work was supported by the National Key Research and Development Program of China (No. 2018YFA0900100), and the National Natural Science Foundation of China (No. 31670081).

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Conflicts of interest

None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in this study.
Table S2. Plasmids used in this study.
Table S3. Strains used in this study.

Data S1. The effect of Rtm on the activity of promoter PrppA. The activities of promoter PrppA treated with different concentrations of antibiotic Rtm were obtained by detecting β-galactosidase activity and calculated from three biologically independent replicates, and shown as the mean ± SD. ns, no significant difference, P > 0.05. Rtm, 0.5 μg/mL (0.965 μM), 0.7 μg/mL (1.352 μM).