Mining and Understanding of New Transcriptional Regulatory Elements from Licorice-Derived Endophyte *Serratia Rubidaea* W12-1

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Abstract. There is an increasing need for precise regulation of complex metabolic pathways in the cell factory development, and thus the discovery of new *cis*-regulatory elements and the responding *trans* elements is especially attracting the attention of scientific researchers. Previously, an endophyte *S. rubidaea* W12-1 was isolated from the root of *Glycyrrhiza uralensis* Fisch. derived from the northwest of China. Here, it was found that prodigiosin, a natural red pigment belonging to the class of alkaloids with multiple pharmacological activities, was produced by *S. rubidaea* W12-1 under specific conditions and growth stages, indicating that there should be interesting inducible promoters and regulatory factors in *S. rubidaea*. Therefore, whole genome sequencing and analysis were operated on *S. rubidaea* W12-1 to map the prodigiosin biosynthesis gene cluster, called *pig* cluster, and its upstream promoter P_{pig}. After DNA pull-down assay and proteomics, 22 transcription factor candidates being expected to interact with P_{pig} were screened out. Among them, AstD, DinB, GlgP, GlmS, FimA, MreB, PchP, RplB, RpoB, RpsD, SucB, TopA, TorZ, TPA, YaaA, YeiP were confirmed to be transcriptional repressors to P_{pig} according to the *in vitro* experiments in which the gene expression vectors (pRS415-P_{gapA}-gene of the transcription factor candidate-P_{pig}-*eGFP*) were constructed and individually transferred into *Escherichia coli* followed by the cultivation and fluorescence detection of the engineered strains. Meanwhile, it was found that L-proline, an upstream compound of prodigiosin metabolism, could significantly enhance the transcriptional repression of SucB and significantly alleviate the transcriptional repression of FimA, TopA and TorZ on P_{pig}-*eGFP*, while malonyl-CoA, a second upstream compound of prodigiosin metabolism, could significantly enhance the transcriptional repression of CgtA and significantly alleviate the transcriptional repression of TorZ and YeiP on P_{pig}-*eGFP*. In summary, this study improved the understanding of the regulation mechanism of prodigiosin metabolism in *Serratia*, provided potential transcriptional regulatory elements in metabolic engineering and high-throughput screening of target strains, and contributed to enriching the genetic components derived from microorganisms in synthetic biology.

1 Introduction

The promoter, which is situated upstream of the coding gene, is capable of specific binding with RNA polymerase to initiate gene transcription [1]. The target gene's expression intensity and efficiency are determined by the promoter's position and sequence composition [2]. Depending on the mode of transcription of the downstream gene turned on by the promoter, promoters can be categorized into constitutive promoters and inducible promoters [3]. In synthetic biology, it is frequently required to choose various promoter types and strengths to precisely control target gene expression following various experimental designs. Given the unique features of inducible promoters that can be induced to turn on or off, they play a very important role in the field of metabolic engineering and synthetic biology, so the mining and supplementation of novel inducible promoters are particularly important [4-5]. A multitude of inducible promoters have been identified in recent years; these induction signals encompass a wide range of compounds with distinct structures, including sodium lauryl sulphate [6], tetracycline [7-8], gallic acid [9], and L-rhamnose [10]. Additionally, there are physical signals, such as oxygen [11], light [12], temperature [13], and dissolved oxygen [14].

As important components in the design of synthetic biology gene circuits, inducible promoters need to be linked with transcription factors to achieve precise regulation of transcriptional expression of downstream target genes. The "promoter-transcription factor" expression system also plays an important role in various fields, and biosensor devices based on the promoter-transcription factor expression system are widely used in

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the fields of medicine and environmental monitoring due to their easy portability and real-time detection. In addition, in the field of biomanufacturing, metabolite concentrations have been linked to visualization signals, such as fluorescence intensity, to develop high-throughput screening platforms, which in turn enable the screening of high-yielding strains [15].

In recent years, research on microorganisms of the genus *Serratia* has been gradually carried out because of the possibility of synthesizing a natural red pigment with good physiological-pharmacological activity, prodigiosin [16]. Investigating the synthesis and metabolism of prodigiosin in the model bacterium *Serratia marcescens* of the species *Serratia marcescens* has been the main focus of the majority of pertinent investigations. The exploration of regulatory mechanisms and the mining of regulatory elements in *Serratia marcescens*, especially in non-model microorganisms, is still an unknown treasure trove that needs to be mined.

2 Methods

2.1 Whole genome sequencing and *pig* gene cluster mining

Serratia rubidaea W12-1 organisms were cultured and collected, and sent on dry ice to Shenzhen Huada Genetics Co. for whole genome sequencing. Three functional annotation methods were used for genome-wide data: KEGG, COG, and GO. After whole genome sequencing annotation, relevant data collection using the EMBL database (https://www.ebi.ac.uk/) and NCBI database (https://www.ncbi.nlm.nih.gov/), followed by local BLAST sequence alignment, the whole genome of *S. rubidaea* W12-1 was mined in the prodigiosin synthesizing *pig* gene cluster and its promoter P_{pig}.

2.2 Analysis of gene expression in the *pig* gene cluster

Group A was the experimental group that had less dissolved oxygen, and Group B was the control group. Using *S. rubidaea* W12-1 in groups A and B, RT-qPCR was used to examine variations in the expression of the genes *pigA*, the first gene on the *pig* gene cluster, and *pigC*, the gene encoding the terminal condensing enzyme PigC, at various time points of 0, 5, 10, and 15 h. The following table displays the findings.

2.3 Heterologous expression vector construction

P_{pig} and the potential transcription factor genes were amplified from *S. rubidaea* W12-1 cells, and constructed into the pRS415 expression vector along with the strong constitutive promoter P_{gapA} and the fluorescent reporter gene *egfp* in *E. coli* by Gibson assembly. Then, the vectors were transferred into *E. coli* Top10 to characterize the effect of potential transcription factors in regulating expression by the expression of the fluorescent protein eGFP. This was done to confirm whether the screened transcription factors had transcriptional regulatory interactions with P_{pig}. The reporter strains established based on combinations of heterologous DNA sequences were named separately, and to exclude the interference of *E. coli* endogenous transcription factors on the P_{pig} initiation strength, an additional *E. coli* reporter strain was constructed that did not contain a potential transcription factor of *S. rubidaea* W12-1 origin in the expression vector, and was named CK.

2.4 Detection of fluorescence intensity

The successfully constructed plasmid was introduced into *E. coli* Top10 and cultured for 12 h. After that, 1 mL of the bacterial solution was taken into a centrifuge tube with 1 mL of PBS and centrifuged at 12000 rpm for 1 min, and the supernatant was poured off. Following the addition of 1 mL of PBS to resuspend the bacterial body, 100 uL of each bacterial solution was transferred to the enzyme label plate. An enzyme marker with an excitation wavelength of 488 nm and an emission wavelength of 507 nm was then used to measure the fluorescence intensity of each bacterial solution. Calculate the fluorescence intensity per cell of different strains after 12 h.

All transcription factor-containing strains were chosen as experimental subjects and L-proline or malonyl-CoA were added exogenously to a final concentration of 5 g/L or 10 mg/L, respectively, to assess whether these substances are important inducers. After the modified bacteria were incubated in liquid fermentation for 12 hours, the intensity of green fluorescence per unit cell was evaluated. As a control, CK bacteria lacking the W12-1-derived transcription factor were employed.

2.5 Statistical analysis of data

Significant differences between the two groups of data were analyzed using the Student's t-test. If p is less than 0.05, it indicates a significant difference between the two data groups; if p is less than 0.005, it indicates a highly significant difference. If p is more than 0.05, there is no significant difference between the two data groups.

3 Results and Discussion

3.1 Genomic analysis of *Serratia rubidaea* W12-1 and acquisition of P_{pig}

Following sequencing, the genome of *Serratia rubidaea* W12-1 was found to contain 4768 genes, have a genomic size of 5.04 Mb, and a GC content of 60.41%. This strain has a significant advantage in amino acid transporter metabolism, transcription and carbohydrate transport and metabolism in terms of gene number enrichment.

The *pig* gene cluster has been identified as a synthetic gene cluster for prodigiosin. Following annotation of the whole genome sequencing and search comparison, it was discovered that *S. rubidaea* W12-1 harbours a *pig* gene cluster. This cluster, approximately 21 kb in length, comprises 14 linearly arranged *pig* genes: *pigA*, *pigB*,

pigC, *pigD*, *pigE*, *pigF*, *pigG*, *pigH*, *pigI*, *pigJ*, *pigK*, *pigL*, *pigM*, and *pigN* (Fig. 1). To build the regulatory system later on, we chose the sequence 300 bp upstream of this gene cluster as P_{pig}, its promoter sequence.

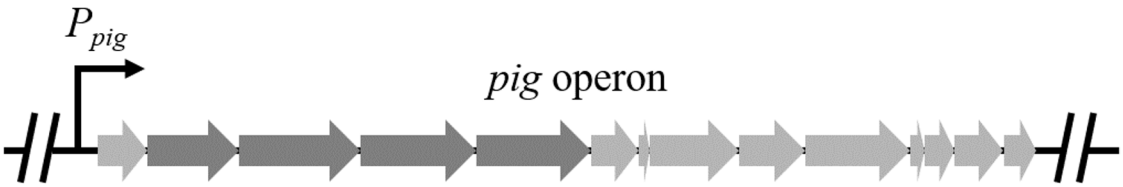

Fig. 1. Prodigiosin biosynthesis gene cluster.

This analysis revealed that *S. rubidaea* W12-1 includes an abundance of regulatory-related genes. Based on genome-wide annotation, we predicted that *S. rubidaea* W12-1 should have a high number of P_{pig}-responsive inducible chemicals and transcription factors to support the regulation of the expression of the *pig* gene cluster.

3.2 Exploration of *pig* gene cluster expression in *S. rubidaea* W12-1

As shown in Fig. 2, in *S. rubidaea* W12-1 sourced from group A, the expression of both *pigA* and *pigC* was low and did not change significantly during 0 - 15 h. At 10 - 15 h of fermentation, the expression level of *pigA* in group B was 8.2-fold of that at 5 h, and that of *pigC* was 46.7-fold of that at 5 h, with a significant elevation in the expression of both *pigA* and *pigC*. It was shown that the onset of prodigiosin synthesis was directly connected with the onset of *pig* gene cluster expression in group B, where dissolved oxygen levels were quite good. This led to the hypothesis that P_{pig} could be an inducible promoter.

In conclusion, *S. rubidaea* W12-1 needs a specific concentration of dissolved oxygen to synthesize prodigiosin, and the initiation of this synthesis is independent of growth, which is linked to the activation of the *pig* gene cluster. It is possible to activate the expression of the *pig* gene cluster in an environment with reasonable ventilation, indicating that P_{pig} is a putative inducible promoter that might work in tandem with cellular endogenous transcription factors to co-regulate the transcriptional turn-on of the *pig* gene cluster.

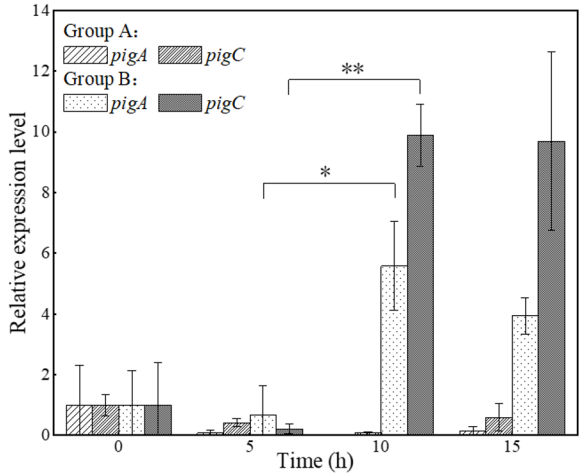

Fig. 2 RT-qPCR analysis of the *pigA* and *pigC* gene expression levels under different culture conditions.

3.3 Mining the "transcription factor-P_{pig}" regulatory system

3.3.1 Mining of transcription factors

To study the transcription factors that interact with P_{pig} to achieve transcriptional regulation of downstream gene clusters, we used the experimental method of DNA pull-down in conjunction with proteomics mass spectrometry analysis. We screened a total of 22 proteins, which include Adk, AstD, AtpD, CgtA, DinB, FimA, GlgP, GlmS, GlyS, LeuS, MreB, PchP, RplB, RpoB, RpsD, SucB, TalA, TopA, TorZ, TPA, YaaA, and YeiP.

3.3.2 Heterologous expression vector-based screening of "transcription factor-P_{pig}" regulatory elements

Out of all the screened transcription factors, only a small number of transcription factors can non-significantly boost P_{pig}'s transcription initiation as transcription activators, as Fig. 3 illustrates. Most transcription factors inhibit the transcriptional initiation of P_{pig} as transcriptional repressors, such as SucB, TopA, TorZ, YeiP, FimA, and CgtA.

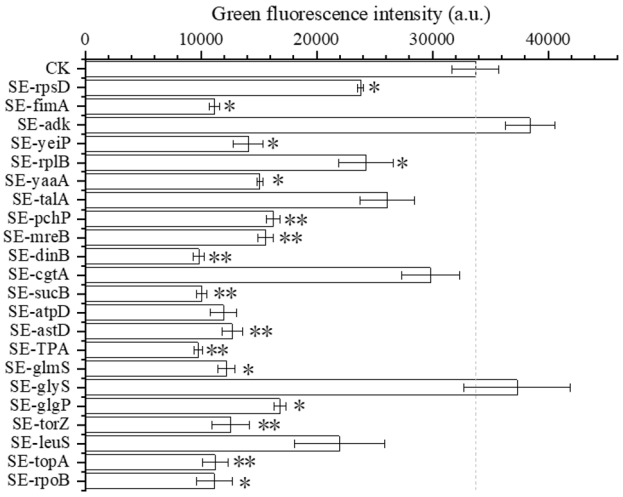

Fig. 3. Fluorescence intensities were measured and normalized by the cell intensities.

3.3.3 Theoretical screening and validation of potential inducible compounds

Considering that the majority of the transcription factors that were excavated were transcriptional repressors, we deduced that *S. rubidaea* W12-1 possessed an endogenous inducible compound. As the culture time was extended, the metabolite accumulated and reached a certain concentration. At this point, the compound interacted with the repressive transcription factors to alter their conformation, releasing the transcriptional repressors from the promoter region and lifting P_{pig}'s repression. This allowed the strain *S. rubidaea* W12-1 to open the spirit of red pigment synthesis. Strain *S. rubidaea* W12-1 starts producing prodigiosin when P_{pig} is not inhibited. We found the suspected inducible compounds that may accumulate as L-proline or malonyl-CoA by expression abundance at the transcriptional level of functional genes.

5 g/L of L-proline dramatically aided in the transcriptional repressor SucB's inhibitory initiation of P_{pig}, as demonstrated in Fig. 4a. L-proline at 5 g/L significantly attenuated the inhibitory initiation of P_{pig} by the transcriptional repressors FimA, TopA, and TorZ.

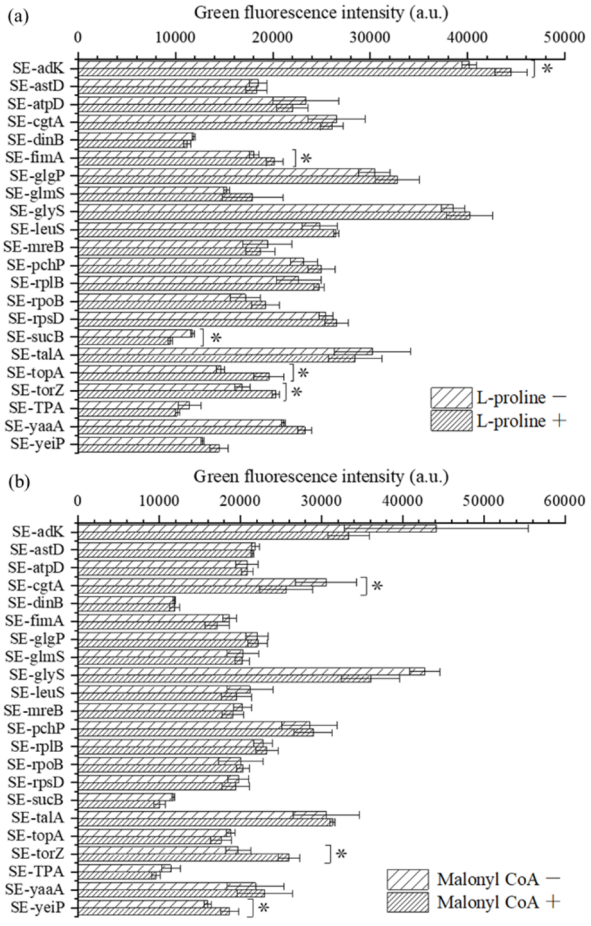

Fig. 4. Fluorescence intensities response to inducible compounds were measured and normalized by the cell intensities.

10 mg/L of malonyl-CoA considerably aided in the transcriptional repressor CgtA's inhibitory initiation of P_{pig}, as demonstrated in Fig. 4b. Malonyl-CoA at 10 mg/L significantly attenuated the inhibitory initiation of P_{pig} by the transcriptional repressors TorZ and YeiP. Once again, it was established that transcriptional repressors, including CgtA, FimA, SucB, TopA, TorZ, and YeiP, can interact and bind with specific substances to perform regulatory tasks.

3.3.4 Inference of the transcriptional regulation mechanism of the pig gene cluster

The synthesis of prodigiosin in *S. rubidaea* W12-1 may be influenced by a variety of transcription factors working in concert in various temporal and geographical situations to carry out transcriptionally regulated activities. Drawing from prior research, as illustrated in Fig. 5, the following regulatory mechanisms associated with P_{pig} are deduced:

With the presence of transcriptional repressors and the accumulation of inducible compounds with the increase of incubation time, the inducible compounds can bind to the transcriptional repressors, thus causing the release of the transcriptional repressors from the P_{pig}, and ultimately, the activation of the transcription of the prodigiosin synthesis gene cluster. SucB, TopA, TorZ, FimA, and YeiP, for instance, are known to significantly suppress P_{pig}'s transcriptional start.

There are transcriptional repressors, and as the incubation period lengthens, the inducible chemicals build up and have the ability to attach to the transcriptional repressors, intensifying the suppression of P_{pig}. As seen in Fig. 5a, the transcriptional repressors SucB and CgtA, for instance, can bind to L-proline and malonyl-CoA, respectively, greatly increasing the inhibitory initiation of P_{pig}.

There are transcriptional repressors, and as the incubation period lengthens, the inducing substance accumulates and can bind to the transcriptional repressor to reduce P_{pig}'s repression. As seen in Fig. 5b, the transcriptional repressors FimA, TopA, and TorZ, for instance, can bind to L-proline, while the transcriptional repressors YeiP and TorZ can bind to malonyl-CoA, both of which greatly reduce the inhibitory initiation of P_{pig}.

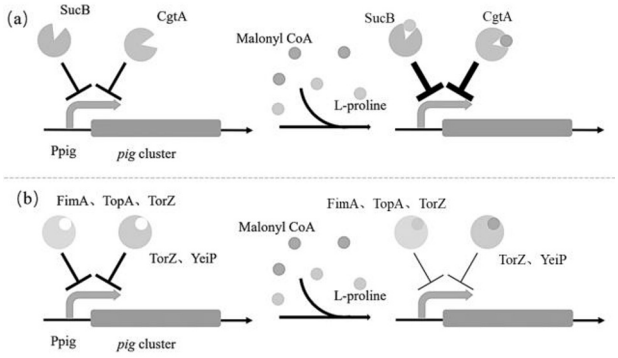

Fig. 5. Possible transcriptional regulatory mechanisms in inducible promoter P_{pig}.

4 Conclusion

The entire genome of *S. rubidaea* W12-1, an endophytic bacterium from the unique environment of *Glycyrrhiza. uralensis* Fisch roots were sequenced in this work. The

prodigiosin-synthesising *pig* gene clusters *pigA*, *pigB*, *pigC*, *pigD*, *pigE*, *pigF*, *pigG*, *pigH*, *pigI*, *pigJ*, *pigK*, *pigL*, *pigM*, and *pigN*, as well as their upstream promoter P_{pig}, were discovered to be present in the strain based on the examination of genome-wide data. It was shown that P_{pig} is an inducible promoter and that the expression of the *pig* gene cluster is not correlated with growth. Experimental screening yielded twenty-two transcription factors that can physically bind to P_{pig}; these transcriptional repressors, AstD, DinB, FimA, GlgP, GlmS, MreB, PchP, RplB, RpoB, RpsD, SucB, TopA, TorZ, TPA, YaaA, and YeiP, can strongly block P_{pig}'s transcription initiation. Furthermore, at a 5 g/L concentration of L-proline, FimA, TopA, and TorZ could considerably reduce the inhibitory initiation effect on P_{pig}, while SucB could dramatically boost the inhibitory effect on the inducible promoter P_{pig}. At a malonyl-CoA concentration of 10 mg/L, TorZ and YeiP greatly reduced the inhibitory initiation of P_{pig}, while CgtA significantly increased the inhibitory effect on the inducible promoter P_{pig}. We were able to identify several transcriptional regulatory expression systems in this investigation, where the induced promoter element is P_{pig}, and the inducing substance is L-proline. This provides a theoretical basis for the rational design of microbial gene circuits for subsequent high-yielding strains (Fig. 6a), as well as also for the screening of engineered strains for high L-proline production based on high-throughput technology (Fig. 6b).

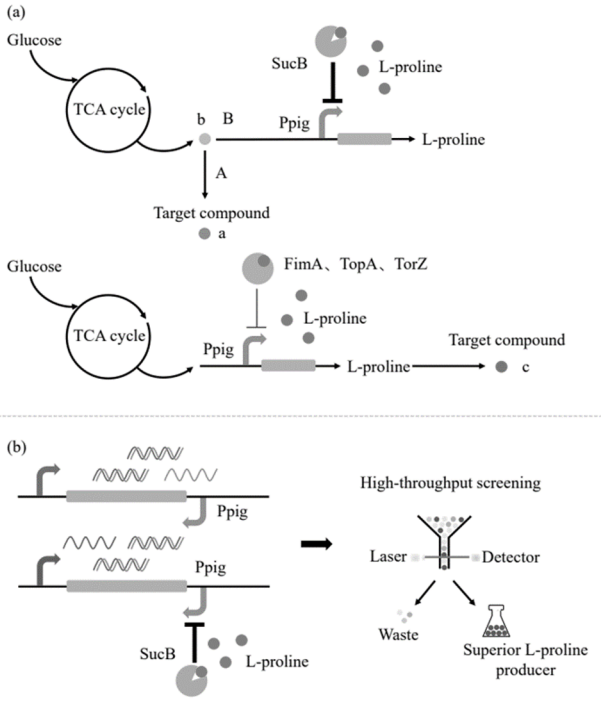

Fig. 6. Application of transcriptional regulatory mechanisms.

In this work, we first suggest and demonstrate that P_{pig}, the *S. rubidaea* W12-1 prodigiosin synthesis promoter, is an inducible promoter. Furthermore, we mined the complete library of transcription factors that can interact with P_{pig} in a variety of culture settings and at different times for the first time. Contribute to enriching the treasure trove of genetic components of microbial origin.

Only the inducible promoter P_{pig}, which is located upstream of the *S. rubidaea* W12-1 cluster of prodigiosin production genes, was examined in this investigation, though. This can be followed up by biosignature analysis and multi-omics linkage to obtain more inducible promoters that can respond to environmental signals/inducers. Extension of the library of synthetic biology components.

Acknowledgments

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