The transcription activator AtxA from Bacillus anthracis was employed for developing a tight-control, high-level, modulable and stationary-phase-specific transcription activity in Escherichia coli

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Abstract

The strong transcriptional activity of the virulent gene pagA in Bacillus anthracis has been proven to be anthrax toxin activator (AtxA)-regulated. However, the obscure pagA transcription mechanism hinders practical applications of this strong promoter. In this study, a 509 bp DNA fragment [termed 509sequence, (−508)−(+1) relative to the P2 transcription start site] was cloned upstream of rbs-GFPuv as pTOL02B to elucidate the AtxA-regulated transcription. The 509sequence was dissected into the −10 sequence, −35 sequence, AT rich tract, SLI/SLII and upstream site. In conjunction with the heterologous co-expression of AtxA (under the control of the T7 promoter), the −10 sequence (TATACT) was sufficient for the AtxA-regulated transcription. Integration of pTOL02F + pTOLAtxA as pTOL03F showed that the AtxA-regulated transcription exhibited a strong specific fluorescence intensity/common analytical chemistry term (OD₆₀₀) of 40 597 ± 446 and an induction/repression ratio of 122. An improved induction/repression ratio of 276 was achieved by cultivating Escherichia coli/pTOL03F in M9 minimal medium. The newly developed promoter system termed P_{AtxA} consists of AtxA, the −10 sequence and Escherichia RNA polymerase. These three elements synergistically and cooperatively formed a previously undiscovered transcription system, which exhibited a tight-control, high-level, modulable and stationary-phase-specific transcription. The P_{AtxA} was used for phaCAB expression for the stationary-phase polyhydroxybutyrate production, and the results showed that a PHB yield, content and titer of 0.20 ± 0.27 g/g-glucose, 68 ± 11% and 1.5 ± 0.4 g/l can be obtained. The positive inducible P_{AtxA}, in contrast to negative inducible, should be a useful tool to diversify the gene information flow in synthetic biology.

Key words: AtxA; stationary-phase promoter; polyhydroxybutyrate

Graphical Abstract

1. Bacillus derived P_{AtxA} is positively regulated by AbxA in E. coli

IPTG

P_{T7}  AtxA  P_{AtxA}  GFPuv

2. P_{AtxA} is a stationary-phase specific promoter

3. P_{AtxA} is tight-control, high-level, and modulable

4. P_{AtxA} is employed in PHB production
1. Introduction

A promoter is a cis-acting DNA sequence recognized by RNA polymerase (RNAP) for subsequent transcription. Promoter activity often involves the interaction of the promoter with RNAP and regulatory factors, including regulatory proteins (activator and repressor) and effector molecules (inducer and corepressor). Promoter activity can be quantified by the probability of promoter occupancy by RNA polymerase based on thermodynamic models (1), and the probability of promoter occupancy by RNAP being proportional to the gene expression is one of the most important assumptions in these models. This assumption facilitates the quantification of promoter activity by monitoring the protein expression level (2). Transcription regulation can be negative inducible or repressible when the repressor protein cooperates with inducers or corepressors, respectively. Additionally, the transcription regulation can be positive inducible or positive repressible when the activator protein cooperates with inducers or corepressors, respectively. Transcriptional regulation usually occurs at the transcription binding and initiation stages. From the application perspective, promoters can be classified as constitutive, inducible or self-inducible. Among these, the inducible type activates with inducers or corepressors, respectively. Transcriptional regulation usually involves the interaction of the promoter with RNAP and regulatory factors, including regulatory proteins (activator and repressor) and effector molecules (inducer and corepressor). Promoter activity can be quantified by the probability of promoter occupancy by RNA polymerase (3). PLac (4, 5), Prr (6) and P1 promoters (7). PRAD is a positive inducible promoter, where the activator protein is AraC and the inducer is arabinose. In contrast, P1, Prr and P2 are negatively inducible, consisting of the Lacl repressor protein and lactose or lactose analogue, isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer.

Bacillus anthracis is a Gram-positive, aerobic and spore-forming Bacillus. B. anthracis, which carries two virulence plasmids, pXO1 (182 kb) and pXO2 (95 kb), is a virulent strain. The first mega plasmid pXO1 carries three virulent genes pagA, lef and cya (encoding cell-binding protective antigen (PA, 85 kDa), lethal factor (LF, 83 kDa) and edema factor (EF, 89 kDa)) (8). A pair of PA and EF proteins forms an edema toxin and that of PA and LF proteins forms a lethal toxin. The mega plasmid pXO2 carries the capBCADE operon for the production of the antiphagocytic poly-D-glutamic acid capsule, protecting the pathogen from phagocytosis. The secretion of toxins and capsules from B. anthracis is considered critical to protect it against the host immune system and to cause infection. The expression of these virulence genes is highly regulated at the transcriptional initiation level by a class of regulators called phosphoenolpyruvate-dependent phosphotransferase regulation domain-containing virulence regulators (PCVRS) (9). The expression of PA, LF and EF is activated by CO2-bicarbonate and temperature (10, 11). For instance, 5% CO2 and 0.8% sodium bicarbonate in medium are optimal for toxin and capsule production (12).

Anthrax toxin activator (AtxA, encoded on pXO1) is a major positive regulator of anthrax toxin and capsule expression (13, 14). Thus, it is a potential target for developing therapeutics for anthrax infection. AtxA is a PCVR that contains two helix-turn-helix (HTH) domains at the N-terminus, two phosphotransferase system regulation domains (PRDs) and one EIIb-like domain at the C-terminus (15). It has been suggested that the homodimeric state of AtxA is the active structure for regulation, and the dimerization of AtxA is positively promoted by a high CO2/bicarbonate level and de-phosphorylation of the EIIb domain and H379 in PRD2 (15, 16). Furthermore, the phosphorylation of H119 in PRD1 is essential for DNA binding (16). All three functional domains of AtxA are commonly found in PCVR; nevertheless, the molecular mechanisms of AtxA in regulating toxin expression are not fully clear. For example, it has been found that transcripts derived from P1 are enhanced 19-fold in a 20% CO2 atmosphere (17). However, it has been also reported that the presence of bicarbonate does not enhance the in vitro binding activity of AtxA to DNA (18).

The pagA gene has two promoters, P1 and P2. The transcription start sites (TSSs) of P1 and P2 transcripts are −58 and −26 relative to the translation initiation codon site of PA (13). P1 is regulated by AtxA (13, 17), and the 90-bp DNA region upstream of the P1 TSS is sufficient for the AtxA-dependent transcription regulation (19). The sequence of the 90-bp DNA region upstream of the P1 TSS is high in AT% and is predicted to form an intrinsic AT-rich curvature structure. The AtxA-specific regulation is DNA-structure-specific rather than sequence-specific (19). In a later study, it was further demonstrated that (−105)−(−67) upstream of the P1 TSS is an essential cis-acting site where the region forms a stem loop structure (and thus named as SLII) (18). The specific interaction between AtxA and SLII is thought to be structure-specific rather than sequence-specific (18). A second curvature is predicted 30-bp downstream of the P1 TSS, and it has been shown that the second curvature is not necessary for the AtxA-dependent transcription regulation (20). Numerous data have been reported for the AtxA-dependent transcription; however, the exact sequence of the P1 promoter has not been fully elucidated. In contrast, P2 is thought to be a constitutive and relatively weak promoter. The −10 and −35 sequences have been suggested, despite the fact that the predicted −35 sequence, TTCCCA, of P2 differs from the consensus sequence, TTGACA, and the space of 20 bp between the predicted −10 and −35 sequence is not optimal. A recent RNA-seq analysis disclosed that transcripts starting from P2 are abundant (21).

Polyhydroxybutyrate (PHB), a family of polyhydroxyalkanoates (PHAs), is eco-friendly and biodegradable. PHB has been proposed as an alternative to polypropylene. Ralstonia eutropha is a naturally PHB-producing strain, and its PHB biosynthesis pathway has been studied in detail (22). The PHB biosynthetic process is initiated by the condensation of two acetyl-coenzyme A (CoA) molecules to produce acetoacetyl-CoA, which is catalyzed by the β-ketothiolase (encoded by phaA). Acetoacetyl-CoA is then reduced to (R)-3-hydroxybutyryl-CoA by the NADPH-dependent acetoacetyl-CoA reductase (encoded by phaB). Finally, PHB is formed by the polymerization of (R)-3-hydroxybutyryl-CoA with PHB synthase (encoded by phaC). The phaCAB genes from R. eutropha have been heterologously cloned into E. coli, and a PHB productivity of 2.08 g l−1 h−1 and a PHB content of 80% were obtained (23). Recombinant E. coli harboring the phaCAB genes from Alcaligenes latus has been reported, and a PHB productivity of 4.63 g l−1 h−1 and a PHB content of 78% were obtained (3). Previously, we have successfully reconstructed PHB biosynthesis pathway of Caldironas manganoxidans in E. coli, where a PHB titer of 16.8 ± 0.6 g/l, a yield of 0.28 g/g-glucose, a content of 74% and a productivity of 0.28 g l−1 h−1 were reported (24). We further constructed an antibiotic- and inducer-free vector for the functional expression of the phaCAB genes of C. manganoxidans in recombinant E. coli, and PHB with a yield of 0.26 ± 0.07 g/g-glucose and content of 44 ± 3% were reported (manuscript submitted).

The promoter responsible for the pagA transcription is strong, as up to 20 mg/l of PA can be obtained when B. anthracis is cultured under optimal conditions (12). This indicates the potential of P1 and/or P2 for industrial applications. In this study, a 509-bp DNA fragment ([−508]−[−1] relative to the P2 TSS, and termed 509sequence in this study), containing P1 and P2, was cloned upstream of rbs-GFPuv to elucidate the AtxA-based transcription regulation. First, the 509sequence was dissected into five genetic elements: the −10 sequence (17, 18), −35 sequence (17, 18), AT-rich tract (19), SLII/SLII (18) and upstream site (Figure 1).
Figure 1. (A) Schematic of pTOL02 series harboring genetic elements in 509seq. (B) Nucleotide sequences of the 509seq with labeled genetic elements. (C) Constructions of pTOL03 series by inserting the AtxA operon in pTOLAtxA into pTOL02 series. The plasmid sizes of pTOL02D, pTOL02E and pTOL02F are 6359, 6293 and 5850 bp, respectively. The plasmid sizes of pTOL03D, pTOL03E and pTOL03F are 8312, 8246 and 7803 bp, respectively.
The regulatory role of each cis-acting element in promoter activity was systematically investigated in conjunction with the heterologous co-expression of AtxA in *E. coli*. While P2 is thought to be a weak and constitutive promoter in *B. anthracis*, this study is the first to prove that the AtxA-regulated transcription in *E. coli* can be independent from P1 and P2. By understanding the interaction among the cis-acting site, cognate RNAP and AtxA, a novel promoter three-hybrid P3H$_{10,AtxA}$ has been first demonstrated, where the hybrid talks of −10 sequence (TATACT), AtxA and host RNAP provide a tight-control, high-level, modulable and stationary-phase-specific expression promoter. In this study, the phaCAB genes of *C. manganoxidans* were cloned downstream of P3H$_{10,AtxA}$ and the stationary-phase PHB production in *E. coli* was examined and reported.

2. Materials and methods

2.1 Construction of pTOL02 and pTOL03 series

pTOL02AtxA, pCDFDuet-533seq and pTOL02A were lab stocks that have been previously cloned in our lab (see supplementary materials for maps and primers used).

To construct pTOL02B, the DNA fragment encoding full-length GFPuv was amplified from pDSK-GFPuv with primers SLIC-F-GFPuv-01 and SLIC-R-GFPuv-01, and the vector fragment was amplified from pTOL02A, a pET29a-based backbone, with primers SLIC-F-pTOL02A-01 and SLIC-R-pTOL02A-01. The assembly of two fragments was achieved by one-step sequence- and ligation-independent cloning (SLIC), as described previously (25, 26). In brief, the vector and insert fragments in deionized (DI) water were mixed at molar ratios of 1:1. One microliter of T4 DNA polymerase (New England Biolabs Inc., MA, USA) was added to 10 μl of the vector/insert mixture and incubated at room temperature for 2.5 min. The reaction mixture was immediately placed in an ice bath for 10 min to inhibit the nuclease reaction while facilitating annealing. This mixture was directly used for the bacterial transformation.

pTOL02C was obtained by deleting SLI/SLII from pTOL02B using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs Inc., MA, USA) with primers DL-F-SLI/II–01 and DL-R-SLI/II–01. The linearized pTOL02B fragment containing the SLI/SLII deletion mutation was generated by polymerase chain reaction (PCR) with pTOL02B as the template and DL-F-SLI/II–01 and DL-R-SLI/II–01 as the primer set. Then, 1 μl of the PCR product was mixed with the kinase, ligase and DpnI enzyme mixture (New England Biolabs Inc., MA, USA) to circularize the linearized DNA fragment.

pTOL02D and pTOL02E were constructed by substitution mutations of pTOL02B and pTOL02C, respectively, using the Q5® Site-Directed Mutagenesis Kit (New England BioLabs Inc., MA, USA). The primer sets of SS-F-ATGC average-01/SS-R-ATGC average-01 and SS-F-ATGC average-01/SS-R-ATGC average-02 were used for pTOL02D and pTOL02E constructions, respectively. The AT-rich tract (ATATCTCTTTTATTTAPATAA, 92% AT) in pTOL02B and pTOL02C was replaced with a random-sequence DNA fragment, ATCG$_{avg}$ (AAGCTTAGAGGATCGAGATCGATCT, 56% AT).

The recombinant plasmid pTOL02F was obtained by subjecting pTOL02E to site-directed mutagenesis deletion (Q5® Site-Directed Mutagenesis Kit, New England BioLabs Inc., MA, USA) with primers DL–F–10 upstream-01/DL–R–10 upstream-01 so that –35 and the upstream site were removed. The resulting pTOL02F containing 4% of the 509 sequence and only the –10 sequence was retained.

The pTOL03 series was obtained by combining the synthetic atxA operon (P$_{r-rbs}$-rbs-atxA-T7 terminator) in the pTOLAtxA with pTOL02 series (Figure 1C). The synthetic atxA operon (1953 bp) was obtained by PCR using primers HF-F-AtxA-01/HF-R-AtxA-01, and the pTOL02 backbone was obtained by PCR with primers HF-F-pTOL02system-01/HF-R-pTOL02system-01. The synthetic atxA operon and the pTOL02 backbone were assembled using the NEBuilder HiFi DNA Assembly Kit (New England BioLabs Inc., MA, USA).

pTOL03FphaCAB was obtained by replacing the rbs-GFPuv in pTOL03F with the rbs-phaCAB gene cluster of *C. manganoxidans* (3). The rbs-phaCAB gene cluster (3725 bp) was obtained by PCR using primers HF-F-phaCAB-01/HF-R-phaCAB01, and the pTOL03F backbone was obtained by PCR with primers HF-F-pTOL03system-01/HF-R-pTOL03system-01. The rbs-phaCAB gene cluster and the pTOL03F backbone were assembled using the NEBuilder HiFi DNA Assembly Kit (New England BioLabs Inc., MA, USA). The rbs sequence is the native rbs sequence of the phaCAB operon in *C. manganoxidans* (3).

Recombinant plasmids were constructed and maintained in *E. coli* DH5α. Plasmid DNA was isolated from *E. coli* by using the Miniprep Purification Kit (QIAGEN, Hilden, Germany). Electroporation (Bio-Rad Gene Pulser, Bio-Rad, California, USA) was used for transformation.

Table 1 lists plasmids used in this study. Table 2 lists primers used in this study.

2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel containing 5% stacking gel and 12% resolving gel (Bio-Rad, CA, USA) was prepared. The pellet was redissolved in lysis buffer (50 mM Tris–HCl, 100 mM NaCl and 50 mg/l lysozyme, pH 8.0) to a final OD$_{600}$ of 10, and cell disruption was performed using ultrasound. The whole-cell, insoluble and soluble fractions were mixed with 4× SDS-PAGE loading sample buffer (Bio-Rad, CA, USA) and were subsequently boiled at 100°C for 10 min. The prepared sample was subjected to SDS-PAGE analysis.

2.3 Fluorescence assay

One milliliter of bacterial solution was collected and centrifuged at 17 000 × g for 3 min at 4°C. The supernatant was decanted, and the bacterial pellet was washed three times with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2H$_2$O and 2 mM KH$_2$PO$_4$) at 4°C. Finally, the bacterial pellet was resuspended in 1 ml of ice-cold PBS for subsequent fluorescence measurements (F-2500, HITACHI, Tokyo, Japan). The excitation, emission, excitation slit, emission slit and photomultiplier voltage were set at 395 nm, 507 nm, 5 nm, 5 nm and 700 V, respectively. The 1-mm fluorescence cuvette was used.

2.4 PHB production in *E. coli*

BL21P/pTOL03FphaCAB

Twenty-five milliliters of fresh lysogeny broth (LB) media in a 250-ml glass flask was inoculated with an overnight culture of *E. coli* BL21P/pTOL03FphaCAB to reach an initial OD$_{600}$ of 0.05. After 6 h cultivation, sterilized glucose and IPTG were added to the final concentration of 10 g/l and 15 μM, respectively. The pH of bacterial culture was measured at 12, 24, 36 and 48 h and adjusted to 7 with 2 N HCl or 2 N NaOH when needed. Note that the genotype of *E. coli* BL21P is *E. coli* BL21(DE3)ΔptsG.
Table 1. Plasmids used in this study

| Plasmid           | Description                                                                 | Ref.                        |
|-------------------|------------------------------------------------------------------------------|-----------------------------|
| pTOLAtxA         | Recombinant plasmid carries atxA (derived from B. anthracis) under the control of T7 promoter, where atxA was cloned at NdeI and XhoI in pCDFDuet-1 vector | This study                  |
| pCDFDuet-533seq   | Recombinant plasmid carries a 533-bp DNA fragment (−508)−(−25) relative to the P2 TSS, derived from B. anthracis, where the 533-bp DNA fragment was cloned at NdeI and XhoI in pCDFDuet-1 vector, see supplemental materials | This study                  |
| pET29a-EGFP      | Recombinant plasmid carries EGFP gene (derived from Aequorea victoria) under the control of the T7 promoter | Lab stock                   |
| pDSK-GFPuv       | Recombinant plasmid carries the GFPuv gene under the control of PphaCAB | This study                  |
| pTOL02A          | pET29a-EGFP derived recombinant plasmid where the T7 promoter is replaced with the 509seq, see supplemental materials | This study                  |
| pTOL02B          | pTOL02B-derived recombinant plasmid where the EGFP gene is replaced with the GFPuv gene | This study                  |
| pTOL02C          | pTOL02B-derived recombinant plasmid carrying the SLI/SLII deletion | This study                  |
| pTOL02D          | pTOL02B-derived recombinant plasmid carrying the substitution mutation ΔAT_{rich}−ATC{average}, ΔT_{rich} is a 25-bp sequence (ATTATCTCTTTTTATTTATATATA) with 92% AT and ATC{average} is a 25-bp sequence (AAGCTTAGAGGATCGAGATCGATCT) with 56% AT | This study                  |
| pTOL02E          | pTOL02B-derived recombinant plasmid carrying the SLI/SLII deletion and substitution mutation ΔAT_{rich}−ATC{average} | This study                  |
| pTOL02F          | pTOL02B-derived recombinant plasmid carrying the gene deletion of the upstream site, SLI/SLII, AT_{rich} tract and −35 sequence | This study                  |
| pTOL03D          | pTOL02D derived, inserting the synthetic atxA operon (P_{T7}−rbs−atxA−T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02D | This study                  |
| pTOL03E          | pTOL02E derived, inserting the synthetic atxA operon (P_{T7}−rbs−atxA−T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02E | This study                  |
| pTOL03F          | pTOL02F derived, inserting the synthetic atxA operon (P_{T7}−rbs−atxA−T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02F | This study                  |
| pTOL03FphaCAB     | pTOL03F derived, replacing the rbs-GFPuv with rbs−phaCAB gene cluster | This study                  |

2.5 PHB quantification by gas chromatography
The gas chromatography (GC) method was used for PHB quantification as described before (27). Briefly, freeze-dried cell (ca. 20 mg), obtained from the bacterial culture solution, was transferred to a clean spiral test tube and 1 ml of DI H2O was added and mixed well by vortexing. After standing and layering, the organic phase was removed and filtered through a 0.2-μm polyvinylidene difluoride (PVDF) filter and then analyzed by GC. The temperatures of the injector and detector were 230 and 275 °C, respectively. The temperature of the column was set at 100 °C and increased to 200 °C at a rate of 10°C/min and maintained at 200 °C for 2 min. The PHB standard (363502) was purchased from Sigma-Aldrich. The PHB content was calculated as follows: PHB concentration (g/l)/biomass concentration (g/l) (%), where the biomass concentration was calculated by dividing freeze-dried cell weight by the culture volume for preparing freeze-dried cell.

3. Results
3.1 AtxA is a positive regulatory activator of E. coli transcription
To examine the effect of AtxA on the GFPuv expression in E. coli, pTOL02B and pTOLAtxA (Figure 1C) were co-introduced into E. coli BL21(DE3). As shown in Figure 2A, BL21(DE3)/02B + AtxA had a significant specific fluorescence intensity (FI/OD600) of 3259 ± 43 when IPTG was added to the final concentration of 0.03 mM. In contrast, the control experiments, including BL21(DE3) and BL21(DE3)/AtxA strains, showed no FI. BL21(DE3)/02B and BL21(DE3)/02B + AtxA (no IPTG induction) showed FI/OD600 of 28 and 0, respectively. Figure 2 indicates that AtxA is a critical positive regulatory activator of transcription in E. coli.

3.2 TAT_{rich} tract is a negative operator site
First, SLI and SLII were removed from pTOL02B to obtain pTOL02C. The AT_{rich} tract in pTOL02B was replaced with a DNA fragment (ATC{average}) that had the same length as a random sequence (56% AT, 14/25 bp, Figure 1) to obtain pTOL02D. Then, pTOL02E was derived from pTOL02B by removing SLI and SLII and replacing the AT_{rich} tract with ATC{average}.

As shown in Figure 3, BL21(DE3)/02C + AtxA had an FI/OD600 of 4058 ± 294, which was comparable to that of BL21(DE3)/02B + AtxA. When AT_{rich} in pTOL02B was replaced with the ATC{average} fragment, BL21(DE3)/02D + AtxA exhibited a marked increase in the FI/OD600 and reached 23 742 ± 209. The effect of SLI/SLII removal and AT_{rich} replacement on the AtxA-regulated transcription was examined by monitoring the strain BL21(DE3)/02E + AtxA, which provided an FI/OD600 of 29 152 ± 2864. Note that 30 μM IPTG was arbitrarily used for AtxA induction, for the moment and the dependence of FI/OD600 on the IPTG introduction will be investigated below to discuss the modularity of P_atxA in responses to IPTG.

The high FI/OD600 of 23 742 ± 209 provided by BL21(DE3)/02D + AtxA was consistent with the SDS-PAGE analysis results, where two strong bands above the marker sizes of 25 and 50 kDa were revealed, which may correspond to GFPuv (27 kDa) and AtxA (56 kDa), respectively (Supplementary Figure S4).

3.3 Removal of the upstream site and −35 sequence revealed that the AtxA-regulated transcription in E. coli is independent of P1 and P2
The effect of the upstream site and −35 sequence on the AtxA-regulated transcription was investigated by constructing pTOL02F (Figure 1). As shown in Figure 4, BL21(DE3)/pTOL02F + AtxA had the highest FI/OD600 of 45 291 ± 8879 among the pTOL02 series, which was 55% higher than that of pTOL02E + AtxA. Moreover,
Table 2. Primers used in this study

| Primer                        | Sequence (5’→3’)                           |
|------------------------------|--------------------------------------------|
| SLIC-F-GFPuv-01              | AGTAAAGGAGAAGAATTTTCAC                     |
| SLIC-R-GFPuv-01              | GGATCTTTATTTGATGACGCTATCATAG              |
| SLIC-F-pTOL02A-01            | GAGCTCTCAAAATAAGATCCGCGCTGCTTAA          |
| SLIC-R-pTOL02A-01            | GTGAAAAGTTCTTCTCCTTTACTCATATG            |
| DL-F-SLI/II-01               | ATACATCTTTTATATATATATATATAT              |
| DL-R-SLI/II-01               | TTAAGAAGACTTTAAAGGACACACAGAAGGAACCAGAAG |
| SS-F-ATGC average-01         | CAGAGATCGATCTTTGAAACTAAAGTTTA            |
| SS-R-ATGC average-02         | ATCTCTACAAATAAGGACACAGAAGGAACCAGAAG |
| DL-F−−10 upstream-01         | ATATATATTTTATATACAAAAGAAGAGGAT           |
| DL-R−−10 upstream-01         | ATACATATGACTAAAGGAGAAGAACCAGAAG          |
| HF-F-AtxA-01                 | CGCCATGCTCGACACAGCGACAGGA               |
| HF-R-AtxA-01                 | CCGGATCGGTCACACAGCGACAGGA               |
| HF-F-pTOL02system-01         | GAAGCAATTGGAATGGGACGCGCCGCC             |
| HF-R-pTOL02system-01         | GCCTCTAGCTGTTAATTTATTTATTTGGTTCCTCAGAG |
| HF-R-pTOL03system-01         | CAGCCGCTGGTTAACAACAGCGACAGGA            |
| HF-R-phaCAB-01               | GATCCGGCTGCTACAAACTAACAGCGACAGGA        |
| HF-R-phaCAB-01               | GTCCTAGTACGAGCGATCGCTAAGAAGAAAGAAGAGGAC |

The removal of the upstream site and −35 sequence in pTOL02F showed that the AtxA-regulated transcription is independent of P1 and P2. Besides, BL21(DE3)/pTOL02F had a low specific fluorescence of 36±6, indicating that the −10 sequence alone was a very weak promoter in E. coli.

The strong expression of GFPuv in BL21(DE3)/pTOL02F+pTOLAtxA indicated that the AtxA-regulated transcription involved three basic elements, which were AtxA, −10 sequence and E. coli cognate RNAP. We further constructed pTOL03F by inserting the P7-rbs-AtxA-ter operon into pTOL02F to verify the minimal elements for the AtxA-regulated transcription in E. coli (Figure 1C). Figure 4 shows that BL21(DE3)/pTOL03F had an FI/OD600 of 44.024±11.09, which was comparable to that of pTOL02F+pTOLAtxA. pTOL03D and pTOL03E were obtained by inserting the P7-rbs-AtxA-ter operon into pTOL02D and pTOL02E, respectively (see below). The complete sequence of pTOL03F can be found in the supplementary data.

3.4 The AtxA-regulated transcription in E. coli is stationary-phase specific

The cis-acting element required for AtxA-dependent GFPuv expression simply consists of a −10 sequence, but no −35 sequence in E. coli. It can be concluded that AtxA-regulated transcription is independent of housekeeping σ70. In fact, the −10 sequence (TATACT) exactly matched that of a σs-dependent promoter, also known as a type III promoter (28), which requires only the −10 sequence.
The effect of NaCl concentration on the (A) bacterial growth and (B) specific FI of E. coli BL21(DE3)/pTOL03D. The induction of AtxA expression was achieved with the 30 μM IPTG when OD_{600} of bacterial cultures reached 0.4–0.6 (−0.9 and −0.5 on the log scale). Errors represent standard deviation with n = 6.

The effect of induction time on (A) the growth and (B) the specific FI of E. coli BL21(DE3)/pTOL02D + pTOLAtxA. The final IPTG concentration of 30 μM was used for the induction of AtxA expression. Errors represent standard deviation with n = 6.

sequence (TATACT) for transcription. The sigma factor σ^s is the starvation/stationary-phase sigma factor, and it is also known as a global regulator for responding to stress conditions, such as hyperosmolarity (29). This study demonstrated that the AtxA-regulated transcription was stationary-phase-specific and was suggested to be σ^s-dependent.

To explore the effects of hyperosmotic conditions on the growth behavior and FI/OD_{600} of E. coli, the growth behavior and FI/OD_{600} of E. coli BL21(DE3)/pTOL03D were investigated in the presence of 0.17, 0.30 and 1.00 M NaCl. As shown in Figure 5A, 0.17 M NaCl (the recipe of the LB media) was optimal for bacterial growth, whereas 0.30 M had a slightly retarded growth. The 0.17 M and 0.30 M NaCl treatments had late-log-phase/stationary-phase transitions around a cultivation time of 3–4 h. Consistently, E. coli BL21(DE3)/pTOL03D started exhibiting fluorescence at 4 h (Figure 5B). In contrast, 1.00 M NaCl had significantly impeded bacterial growth. Due to this, E. coli BL21(DE3)/pTOL03D reached the late-log-phase/stationary-phase transition at ~7 h. In accordance with the late-log-phase/stationary-phase transition, E. coli BL21(DE3)/pTOL03D showed no fluorescence until 7 h (Figure 5B).

3.5 The AtxA-regulated transcription is tight-control and modulable

The AtxA-regulated transcription was subsequently tested for its background expression by cultivating E. coli BL21(DE3)/pTOL03E and E. coli BL21(DE3)/pTOL03F without IPTG supplementation. Table 3 shows that both pTOL03E and pTOL03F had low FI/OD_{600}s of 155 ± 1 and 333 ± 10, respectively. Thereafter, the FI/OD_{600}s of both strains were investigated to determine the dependence on IPTG concentration. The FI/OD_{600} of pTOL03E had a linear relationship with the IPTG concentration and reached a saturation
value of 44.1 ± 247 at 200 μM (Figure 7A). Therefore, the induction/repression ratio was calculated as 285 (Table 3). The FI/OD\textsubscript{600} of pTOL03F also had a linear relationship with the IPTG concentration; however, it was more sensitive to the IPTG concentration, compared to that of pTOL03E (Figure 7B). The FI/OD\textsubscript{600} showed a saturation value of 40,597 ± 446 at 15 μM IPTG. pTOL03F had an induction/repression ratio of 132, in the same order as pTOL03E, whereas the IPTG dosage was lower by one order of magnitude (Table 3). The complete induction of pTOL03E and pTOL03F was accompanied by an acceptable OD\textsubscript{600} magnitude (Table 3). Although the induction/repression ratio cannot be defined for a constitutive promoter, the basal FI/OD\textsubscript{600} level of pDSK-GFPuv could still be perceived when the bacterial culture first entered the log phase at 2–2.5 h, where the FI/OD\textsubscript{600} values were in a range of 1747–6805. The AtxA-regulated transcription had a better performance concerning strength and basal level expression compared to pDSK-GFPuv.

### 3.6 PHB production in E. coli BL21P/pTOL03FphaCAB

To achieve the stationary-phase PHB production, E. coli BL21P/pTOL03FphaCAB was first aerobically cultivated in LB for 6 h, followed by the supplementation of glucose and IPTG to reach the concentrations of 10 g/l and 15 μM, respectively. It is shown in Figure 8 that a PHB yield, content and titer of 0.20 ± 0.27 g/g-glucose, 68 ± 11% and 1.5 ± 0.4 g/l can be obtained at cultivation time of 12 h with the glucose consumption of 7.3 ± 3.5 g/l (data not shown). When glucose was completely consumed, a PHB yield, content and titer of 0.19 ± 0.0 g/g-glucose, 58 ± 4% and 1.9 ± 0.3 g/l can be obtained (see the time mark of 24 h in Figure 8).

### 4. Discussion

The transcription of pagA (encoding PA toxin protein) in B. anthracis has two promoters, P1 and P2. It has been considered that the P1 promoter is subjected to AtxA regulation while the P2 promoter is constitutive. Extensive studies have been conducted.
to elucidate the AtxA regulation mechanism, including biochemical studies of AtxA and identification of sequence- and/or structure-specific cis-acting sites. Nevertheless, the complexity of the pagA transcription makes results among literature inconsistent. Besides, the sequence for P1 promoter has not been identified.

To avoid the complex and hierarchical regulation of the pagA transcription in B. anthracis, the trans-acting AtxA and cis-acting 509sequence, containing P1 and P2, were reconstituted in E. coli to study the AtxA-regulated transcription and seek practical applications. AtxA was found to be a transcriptional activator in E. coli that actively facilitated transcription (as shown in Figure 1). This finding contrasted with the concept proposed by Toyomane et al., which states that AtxA acts as an inducer to release the suppression (20).

The minimal AtxA-regulated promoter contains SLII (19), and SLII and AtxA binding is specific (18); however, the present study showed that SLII/AtxA removal increased the strength of the AtxA-regulated transcription. These data suggest that SLII/AtxA binding actively downregulated the transcription activity in E. coli. This also suggests that the interaction between AtxA and Escherichia RNAP determines the AtxA-regulated transcription.

Two AT$_{rich}$ tracts upstream and downstream of the P2 TSS site were predicted to form a curvature conformation and were recognized by the DNA-binding protein harboring the HTH domain, such as AtxA, which has a winged-helix DNA-binding domain. Therefore, the AT$_{rich}$ tract is considered to be an essential cis-acting element for the AtxA-mediated regulation in B. anthracis (18, 19). Later, Toyomane et al. postulated that the AT$_{rich}$ tract downstream of the P2 TSS site was a negative operator while the first AT$_{rich}$ tract had no significant role as a cis-acting site for the AtxA-mediated regulation (20). In our study, the promoters in pTOL02F and pTOL03F did not contain the downstream AT$_{rich}$ tract; therefore, the effect of the downstream AT$_{rich}$ tract on promoter activity could not be concluded. However, our study was the first to prove that the first AT$_{rich}$ tract, more specifically, the 25-bp sequence upstream of the −35 box (Figure 1), is the most critical repressor operator. Removal of the first AT$_{rich}$ tract from the 509sequence greatly increased the AtxA-regulated transcription activity in E. coli (Figures 3 and 5). In general, the AT$_{rich}$ tract can be a strong binding target for the histone-like nucleoid-structuring (H-NS) protein in E. coli. The binding of the AT$_{rich}$ tract and H-NS protein prevents RNAP from binding to the promoter and is a common transcriptional control found in core gene transcription. AT$_{rich}$ tract and H-NS protein binding can also act as a xenogeneic silencer, preventing the expression of horizontally acquired genes (32, 33). In this study, the extreme burden of the AT$_{rich}$ tract on the AtxA-regulated transcription in E. coli was governed by H-NS proteins through negative regulation. Although Toyomane et al. have explained the role of H-NS protein binding in the AtxA-regulated transcription in E. coli and have concluded that the cis-acting site is the second AT$_{rich}$ tract (20).

Promoter activity is a function of cis-acting sites, RNAPs and regulatory factors. Several promoter systems, including $P_{BAD}$ (3, 34) and $P_{TT}$ (35), and their working principles have been identified and reconstituted in native or non-native hosts. These tool arrays contain their own orthogonality in host strains and compromise cellular burdens, precise modularity, dynamic control (36, 37) and promoter strength (38, 39). Many studies have focused on RNAP for better control and usage of the promoters. For instance, T7 RNAP has been fragmented, and each fragment is expressed at different levels for better control (35). A new orthogonal tool box was developed in E. coli by hybridizing Bacillus subtilis sigma factors with E. coli apo-RNAP so that this hybridized holoRNAP can recognize the promoter sequence from B. subtilis (40). The development of regulatory factors for transcription control is also under the scientific focus. One critical example is the employment of clustered regularly interspaced short palindromic repeats (CRISPR), thus causing CRISPR interference and CRISPR activation. By taking advantage of CRISPR gene targeting, a multiplex modulation of transcription can be achieved (41). Another interesting regulator design is an RNA-only technology called small transcription activating RNA with a dynamic range of 94-fold and maximum specific fluorescence (superfolder GFP) of ∼2.6 × 10$^6$ (42).

While the promoter, RNAP and regulation factor were three typical elements that constitute the promoter system, the −10 sequence (TATACT), cognate RNAP and AtxA exhibited a new transcription mechanism, where the synergistic and cooperative talks of three provided a tight-control, high-level, modular and stationary-phase-specific expression promoter. This study hypothesizes a possible mechanism that involves two interactions. The first was that between Escherichia RNAP and −10 sequence, where the −10 sequence (TATACT) from Bacillus could barely be recognized by Escherichia RNAP. This became the basis for the tight control of the AtxA-regulated transcription. The second is a possible interaction between Bacillus AtxA and Escherichia RNAP, in which AtxA acted as an activator for the resulting strong promoter activity. More specifically, the employment of the −10 sequence resulted in strong promoter activity specifically at the stationary phase. The detailed mechanism of $P_{AtxA}$ at the molecular level could be further investigated. With the simplicity of the $P_{AtxA}$, which involves three transcriptional elements, the employment of $P_{AtxA}$ in different hosts, following the central dogma of biology, is expected to have minimum toxicity and can be decoupled from the potential regulation restriction. Interestingly, most promoters used in synthetic biology are negative inducible (43); the positive inducible $P_{AtxA}$ should be a useful tool to diversify the gene information flow in synthetic biology. While the orthogonality of hybridized holo-RNAP has been demonstrated that determines the initiation of the promoter activity at the certain cellular state (40), attention to the future development of $P_{AtxA}$ can be genetically and biochemically focused on the activation mechanism of AtxA or other PCVRs. More in vitro nonspecific binding activity can be found in the examples of CI and Cro repressors (44).
Another interesting DNA fragment of 509 sequence was the upstream site/−35 sequence, as seen in pTOL03E. The role of the upstream site/−35 sequence in the AtxA-regulated transcription is clear, which titrates the AtxA activation activity. Compared to pTOL03F, pTOL03E was less sensitive to the AtxA induction (Figure 7). The comparable saturation values of the FI/OD₆₀₀ in pTOL03E and pTOL03F indicated that the titration effect of the upstream site/−35 sequence in pTOL03E followed the competitive kinetics (Figure 7). Another result supporting the titration effect of the upstream site/−35 sequence is the low background of protein expression. While pTOL03F had an FI/OD₆₀₀ of 333 ± 10 at 0 mM IPTG, pTOL03E had an FI/OD₆₀₀ of 155 ± 1. An operator sequence with a titration function has found its application in the modulation of promoter activity. A recent study has reported that the supply of a short decoy DNA sequence of transcription factor binding sites titrates the activity of transcription factors and thus the sensitivity of transcription activity to the transcription factor is changed (45). Note that pTOL03D, harboring SLI/SLII, had an FI/OD₆₀₀ of 160 ± 4 at 0 mM IPTG (data not shown).

The cognate RNAP in P₆00 can rule out the dependence of σ⁷₀ because the −35 sequence is not necessary for P₆00. Instead, it is the σ⁵ that is involved in P₆00 for the following arguments. First, P₆00 is a stationary-promoter specific. Second, the −10 sequence of TATACT used in P₆00 is known as the type III promoter (28) and can be recognized by σ⁵. Third, the dependence of recombinant protein induction on high concentration of NaCl indicates the significant role of σ⁵ (29). The strong activity of P₆00 confirms that sigma factors other than σ⁷₀ can be used in applications. This is interesting because the intracellular concentrations of σ⁷₀ is one order of magnitude lower than that of σ⁷₀ at the log phase and is half of that of σ⁷₀ (46). Moreover, the in vitro data have shown that the binding affinity of σ⁵ to the core RNAP is only half of that of σ⁷₀ (47). The role of σ⁵ in P₆00 should be thoroughly investigated, especially σ⁵ is a global regulator in the stationary phase.

Stationary-phase protein expression is of interest in engineering applications, especially for toxic protein overexpression. However, promoters that can be used for stationary-phase protein production often suffer from the low activity (48). Promoter activity at the stationary phase can be improved by random mutagenesis of the promoter region to isolate the mutant, and the promoter can be increased by 16-fold to reach ~3500 FI [GFPuv]/OD₆₀₀ (49). The development of P₆00 is one way to achieve an accurate and strong protein expression in the stationary phase while inflicting the minimum adverse effect on bacterial growth. For example, a high FI/OD₆₀₀ of 40.68 ± 4.46 for E. coli BL21(DE3)/pTOL03F (Figure 7) had an OD₆₀₀ of 4.0, which was comparable to the control experiments of E. coli BL21(DE3) in LB which had OD₆₀₀ of 4.1 at 9 h (data not shown).

In summary, a promoter three-hybrid has been developed, involving the −10 sequence (TATACT), Bacillus AtxA and Escherichia RNAP. The promoter three-hybrid provides a tight-control, high-level, modulable and stationary-phase-specific transcription activity and was decoupled from the complex regulation restriction in B. anthracis. P₆00 requires 15 μM IPTG for full promoter activity. A high induction/repression ratio of 230 was achieved when M9 minimal medium with 20 g/l glucose was used. The 25-bp AT rich tract was a strong negative operator for P₆00, and SLI/SLII was a minor negative operator. The upstream site/−35 sequence may act as a decoy and titrate AtxA activity as a transcription activator.

In addition to the recombinant protein expression, the capability of P₆00 was investigated in the application of metabolic engineering, as shown in Figure 8. Bacterial culture at the stationary phase is considered a non-growth culture, yet glucose consumption and metabolisms still function (50). Secondary metabolites are specifically biosynthesized at the stationary phase, and improved bioprocess productivity may be obtained when a non-growth culture is used (51). This study demonstrated a stationary-phase PHB production by using P₆00 and the late addition of glucose at fermentation time of 6 h. The feasible results (Figure 8) were in accordance with previous literature that non-growth cultures can be employed in biotechnology (51). Improved performance of E. coli BL21P/pTOL03FphaCAB for PHB production can be expected when the carbon flow can be directed to the PHB biosynthesis specifically in the stationary phase as described in previous studies (52, 53) or employing fed-batch fermentation. One major advantage of stationary-phase biochemical production is that the bacterial growth and the heterologous metabolic pathway are decoupled. While a late addition of glucose was employed in this study, a parallel study was conducted where glucose was added at the beginning. The results showed that a low PHB yield of 0.10 ± 0.0 g/g-glucose was obtained (data not shown). The low PHB yield resulted in a PHB content of 32 ± 2%. The low PHB yield was because a significant amount of glucose was converted to acetic acid during the log phase (data not shown). In contrast, the high PHB yield of 0.19 ± 0.0 g/g-glucose in Figure 8 indicates that glucose was majorly converted into PHB during the stationary phase. In fact, the PHB content reported in this study was better than the one using a continuous promoter for PHB production using the same phaCAB cluster, where the content was 44 ± 3% (article accepted in Frontiers in Bioengineering and Biotechnology). This indicates that when the bacterial growth and heterologous metabolic pathway are decoupled, the balance of the bacterial growth and bio-based chemical production may be of secondary concern when constructing the microbial chassis. This concept can be studied in the future with P₆00 developed in this study.

**Supplementary data**

Supplementary data are available at SYNBIIO online.

**Data availability**

All data could be found in paper or supplementary data. Additional data could be requested from the corresponding authors. Material/plasmids are available upon request to the authors with a material transfer agreement.

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