

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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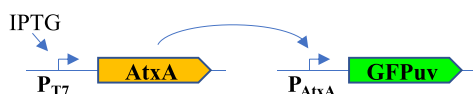
Abstract

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 $40\,597 \pm 446$ 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, $68 \pm 11\%$ 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.

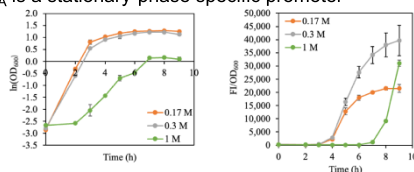
Key words: AtxA; stationary-phase promoter; polyhydroxybutyrate

Graphical Abstract

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

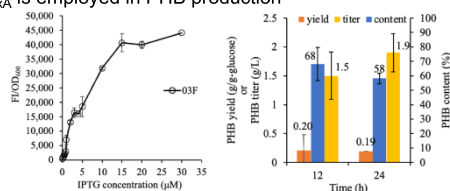


2. P_{AtxA} is a stationary-phase specific promoter



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

4. P_{AtxA} is employed in PHB production



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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 negative 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 has been widely adopted in vector development, including P_{BAD} (3), P_{LAC} (4, 5), P_{T7} (6) and P_L promoters (7). P_{BAD} is a positive inducible promoter, where the activator protein is AraC and the inducer is arabinose. In contrast, P_{Lac} and P_{T7} are negatively inducible, consisting of the LacI 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 virulence 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 H199 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 *Caldimonas manganoxidans* in *E. coli*, where a PHB titer of 16.8 \pm 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 \pm 0.07 g/g-glucose and content of 44 \pm 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), SLI/SLII (18) and upstream site (Figure 1).

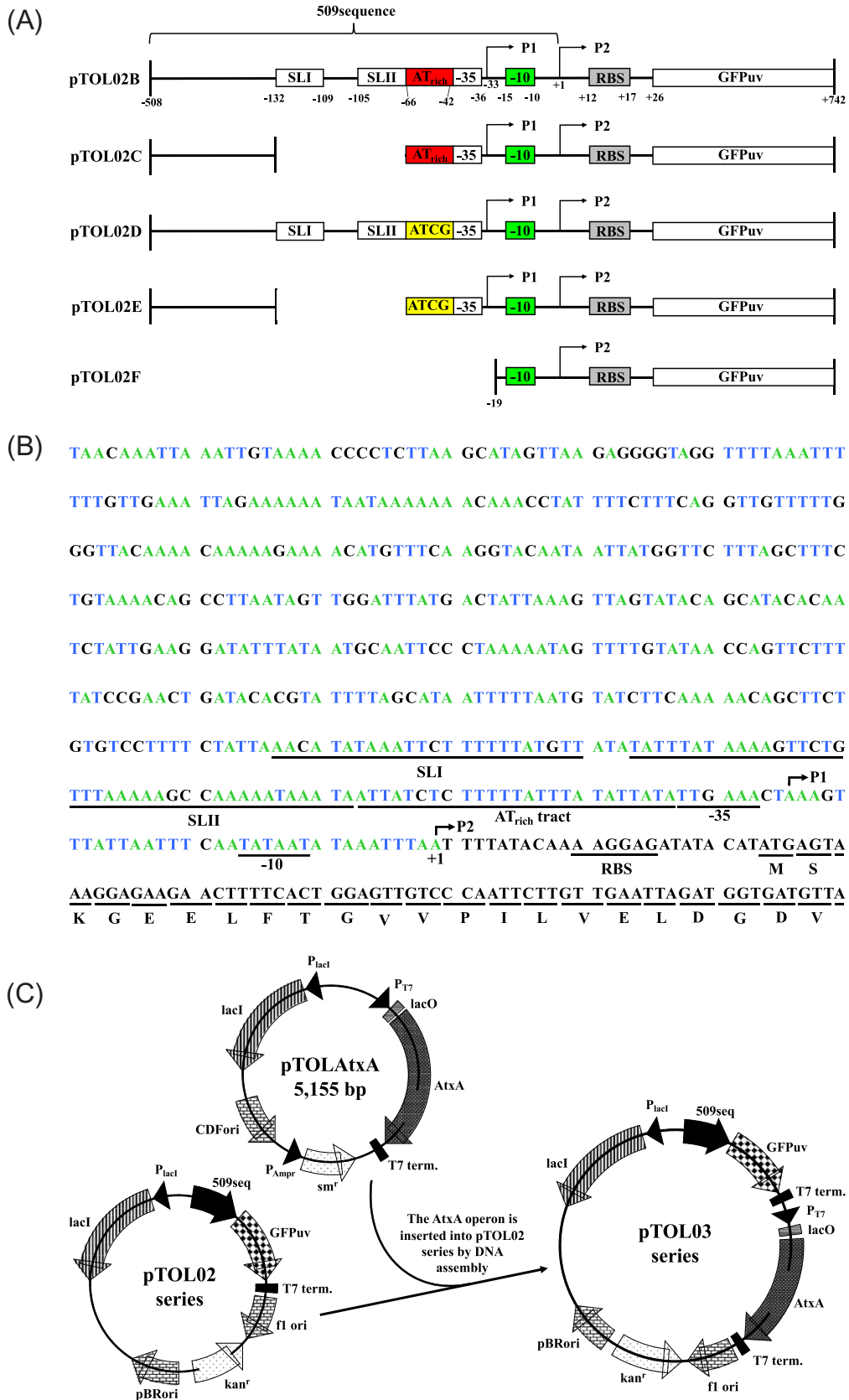


Figure 1. (A) Schematic of pTOL02 series harboring genetic elements in 509seq. (B) Nucleotide sequences of the 509seq with labeled genetic elements. (C) Constructions of pTOL03 series by inserting the AtxA operon in pTOLAtxA into pTOL02 series by DNA assembly. The plasmid sizes of pTOL02D, pTOL02E and 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 μ l 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 μ l 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 while removing the pTOL02B template.

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 (ATTATCTCTTTTATTATTTATTTATA, 92%AT) in pTOL02B and pTOL02C was replaced with a random-sequence DNA fragment, ATCG_{avg} (AAGCTTAGAGGATCGAGATCGATCT, 56%AT).

The recombinant plasmid pTOL02F was prepared 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 509sequence and only the -10 sequence was retained.

The pTOL03 series was obtained by combining the synthetic *atxA* operon (P_{T7}-*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-*phaCAB*01, 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* DH5 α . 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 OD₆₀₀ of 10, and cell disruption was performed using ultrasound. The whole-cell, insoluble and soluble fractions were mixed with 4 \times 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 $\times g$ 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 Na₂HPO₄ · 2H₂O and 2 mM KH₂PO₄) 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 OD₆₀₀ of 0.05. After 6 h cultivation, sterilized glucose and IPTG were added to the final concentration of 10 g/l and 15 μ M, 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) Δ ptsG.

Table 1. Plasmids used in this study

Plasmid	Description	Ref.
pTOLAtxA	Recombinant plasmid carries <i>atxA</i> (derived from <i>B. anthracis</i>) under the control of T7 promoter, where <i>atxA</i> 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 <i>B. anthracis</i>], 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 <i>Aequorea victoria</i>) under the control of the T7 promoter	Lab stock
pDSK-GFPuv pTOL02A	Recombinant plasmid carries the GFPuv gene under the control of P _{PsbA} . pDSK519 backbone pET29a-EGFP derived recombinant plasmid where the T7 promoter is replaced with the 509seq, see supplemental materials	This study
pTOL02B	pTOL02A-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 with the substitution mutation $\Delta AT_{rich}::ATCG_{average}$. AT_{rich} is a 25-bp sequence (ATTATCTCTTTTATTATATTATA) with 92% AT and $ATCG_{average}$ is a 25-bp sequence (AAGCTTAGAGGATCGAGATCGATCT) with 56% AT	This study
pTOL02E	pTOL02B-derived recombinant plasmid carrying the SLI/SLII deletion and substitution mutation $\Delta AT_{rich}::ATCG_{average}$	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 <i>atxA</i> operon (P _{T7} -rbs- <i>atxA</i> -T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02D	This study
pTOL03E	pTOL02E derived, inserting the synthetic <i>atxA</i> operon (P _{T7} -rbs- <i>atxA</i> -T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02E	This study
pTOL03F	pTOL02F derived, inserting the synthetic <i>atxA</i> operon (P _{T7} -rbs- <i>atxA</i> -T7 terminator) of pTOLAtxA into the downstream of GFPuv of pTOL02F	This study
pTOL03FphaCAB	pTOL03F derived, replacing the rbs-GFPuv with rbs'- <i>phaCAB</i> 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 chloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid were added. The tube was incubated in a water bath for 140 min at 80°C and cooled down to room temperature. Then 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- μ m 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/OD₆₀₀s 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 (ATCG_{avg}) 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 ATCG_{avg}.

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 ATCG_{avg} fragment, BL21(DE3)/02D + AtxA exhibited a marked increase in the FI/OD₆₀₀ and reached 23742 ± 2009. 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 29152 ± 2864. Note that 30 μ M 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 the IPTG.

The high FI/OD₆₀₀ of 23742 ± 2009 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 45291 ± 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	<u>AGTAAAGGAGAAGAAGCTTTTCAC</u>
SLIC-R-GFPuv-01	<u>GGATCTTATTTGTAGAGCTCATCCATG</u>
SLIC-F-pTOL02A-01	<u>GAGCTCTACAAATAAGATCCGGCTGCT</u> <u>AACAAAGC</u>
SLIC-R-pTOL02A-01	<u>GTGAAAAGTTCTTCTCCTTTACTCATATG</u> <u>TATATCTCCTTTTG</u>
DL-F-SLI/II-01	<u>ATTATCTCTTTTATTTATATATATAT</u> <u>TGAAAC</u>
DL-R-SLI/II-01	<u>TAATAGAAAAGGACACAGAAG</u>
SS-F-ATGC average-01	<u>CGAGATCGATCTTTGAAACTAAAGTTTA</u> <u>TTAATTTCAATATAATATAAATTTAAT</u> <u>TTTATAC</u>
SS-R-ATGC average-01	<u>ATCCTCTAAGCTTTATTTATTTTTGGCTT</u> <u>TTTAAACAGAAGC</u>
SS-R-ATGC average-02	<u>ATCCTCTAAGCTTTAATAGAAAAGGA</u> <u>CACAGAAG</u>
DL-F- -10 upstream-01	<u>ATAAATTTAATTTTATACAAAAGGAGAT</u> <u>ATACATATGAGTAAAGGAGAAGAAGC</u> <u>TTTCACTGG</u>
DL-R- -10 upstream-01	<u>ATTATATTGACGCCGACGCATCGTGCC</u>
HF-F-AtxA-01	<u>CCGGATTGGCTCACCACAGCCAGGA</u> <u>TCCGA</u>
HF-R-AtxA-01	<u>CGTCCCATTCCAATTCGTTCAAGCC</u> <u>GAGGG</u>
HF-F-pTOL02system-01	<u>GAACGAATTGGAATGGGACGCGCCC</u> <u>TGTAG</u>
HF-R-pTOL02system-01	<u>GCTGTGGTGAGCCAATCCGGATATAGT</u> <u>TCCTC</u>
HF-F-pTOL03system-01	<u>GATCCGGCTGCTAACAAAGC</u>
HF-R-pTOL03system-01	<u>TTGTATAAAATTAATTTATATTTATATTT</u> <u>GACGCGGAC</u>
HF-F-phaCAB-01	<u>AATATAAATTTAATTTTATACAAGAGGAA</u> <u>CAGATGAAGCGAACCAC</u>
HF-R-phaCAB-01	<u>GCTTTGTAGCAGCCGATCTTAC</u> <u>CCCATGTGCAAGCCGC</u>

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 P_{T7} -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 $44,024 \pm 1109$, which was comparable to that of pTOL02F + pTOLAtxA. pTOL03D and pTOL03E were obtained by inserting the P_{T7} -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 σ^{70} . In fact, the -10 sequence (TATACT) exactly matched that of an σ^s -dependent promoter, also known as a type III promoter (28), which requires only the -10

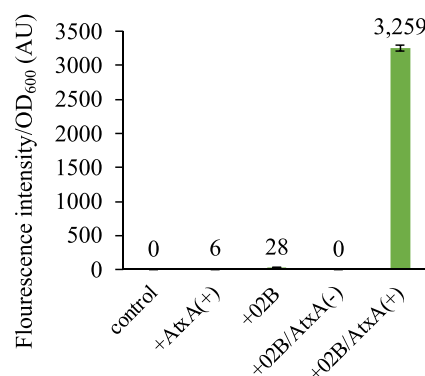


Figure 2. The specific FI of *E. coli* BL21(DE3) strains harboring pTOL02B, pTOLAtxA or pTOL02B/pTOLAtxA combination. The control represents *E. coli* BL21(DE3) and (-) symbol represents no IPTG induction while the (+) symbol represents the IPTG supplementation to the final concentration of 0.03 mM at the cultivation time of 2 h. The bacterial samples were collected at cultivation time of 12 h for FI measurements, where OD₆₀₀ values were in the range between 3.5 and 4.5. Errors represent standard deviation with $n = 3$.

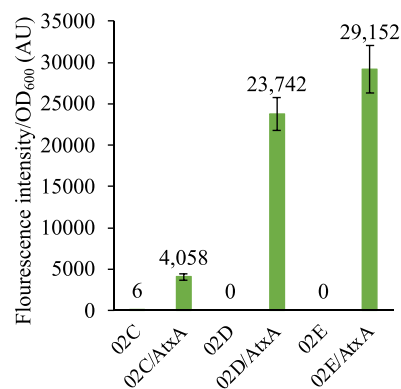


Figure 3. The specific FI of *E. coli* BL21(DE3) strains harboring pTOL02C, pTOL02C/pTOLAtxA, pTOL02D, pTOL02D/pTOLAtxA, pTOL02E or pTOL02E/pTOLAtxA. All strains were subjected to the IPTG supplementation to the final concentration of 0.03 mM at the cultivation time of 2 h. The bacterial samples were collected at cultivation time of 12 h for FI measurements, where OD₆₀₀ values were in the range between 3.5 and 4.5. Errors represent standard deviation with $n = 3$ for pTOL02C and pTOL02C + pTOLAtxA; and $n = 15$ for pTOL02D, pTOL02D/pTOLAtxA, pTOL02E and pTOL02E/pTOLAtxA.

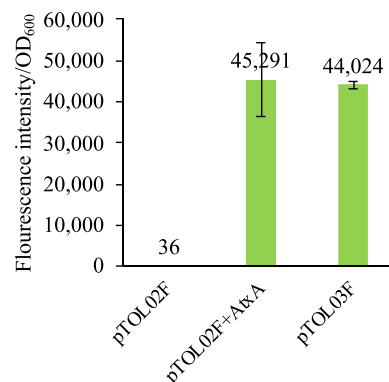


Figure 4. The specific FI of *E. coli* BL21(DE3) strains harboring pTOL02F and pTOL02F/pTOLAtxA. All strains were subjected to the IPTG supplementation to the final concentration of 0.03 mM at the cultivation time of 2 h. The bacterial samples were collected at cultivation time of 12 h for FI measurements. Errors represent standard deviation with $n = 6$ for pTOL02F, $n = 15$ for pTOL02F + AtxA and $n = 3$ for pTOL03F.

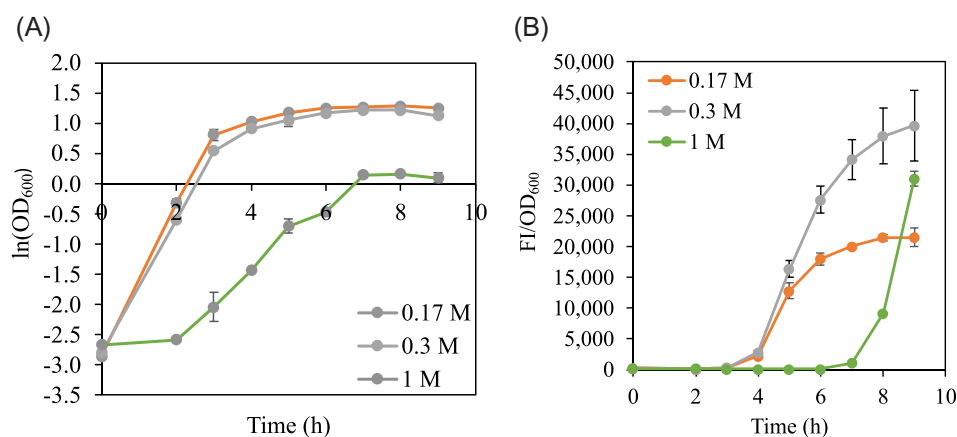


Figure 5. The effect of NaCl concentration on the (A) bacterial growth and (B) specific FI of *E. coli* BL21(DE3)/pTOL03D. The induction of AtxA expression was achieved with the 30 μM IPTG when OD₆₀₀ of bacterial cultures reached 0.4–0.6 (–0.9 and –0.5 on the log scale). Errors represent standard deviation with $n=6$.

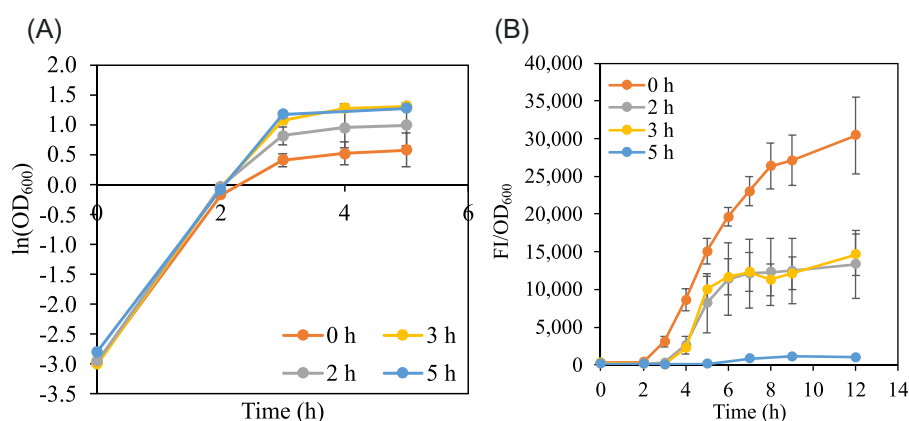


Figure 6. The effect of induction time on (A) the growth and (B) the specific FI of *E. coli* BL21(DE3)/pTOL02D + pTOLAtxA. The final IPTG concentration of 30 μM was used for the induction of AtxA expression. Errors represent standard deviation with $n=6$.

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/OD₆₀₀ of *E. coli*, the growth behavior and FI/OD₆₀₀ 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). The FI/OD₆₀₀s in the hyperosmolarity conditions of 0.30 M and 1.00 M at 9 h were 39654 ± 5731 and 31033 ± 1187 , respectively, higher than that of the 0.17 M NaCl treatment (21515 ± 1537).

The stationary-phase-specific protein expression was supported by investigating the AtxA induction time. When the supplementation of 0.03 mM IPTG for AtxA expression was

supplemented at 2 and 3 h, which is the time when *E. coli* BL21(DE3)/pTOL02D + pTOLAtxA reached the late-log-phase/stationary-phase transition stage (Figure 6A), the fluorescence was revealed instantly (Figure 6B). In contrast, IPTG supplementation at 0 h was a burden for bacterial growth and accelerated the onset of entry into the stationary phase (Figure 6A). Consistently, the bacterial culture exhibited fluorescence at 3 h, which was also in accordance with the stationary-phase expression. Figure 6 shows that the induction at the stationary phase at 5 h was too late for protein overexpression.

While the AtxA and –10 sequence are two genetic elements for the AtxA-regulated transcription, it is suggested that RNAP(σ^s) is the cognate RNAP responsible for the AtxA-regulated transcription.

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

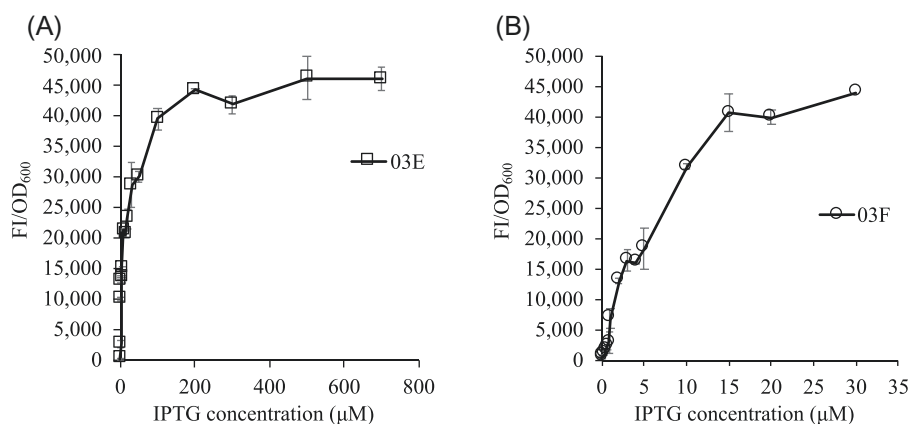
The AtxA-regulated transcription was subsequently tested for its background expression by cultivating *E. coli* BL21(DE3)/pTOL03E and *E. coli* BL21(DE3)/pTOL03F without IPTG supplementation. Table 3 shows that both pTOL03E and pTOL03F had low FI/OD₆₀₀s of 155 ± 1 and 333 ± 10 , respectively. Thereafter, the FI/OD₆₀₀s of both strains were investigated to determine the dependence on IPTG concentration. The FI/OD₆₀₀ of pTOL03E had a linear relationship with the IPTG concentration and reached a saturation

Table 3. Performance of OD₆₀₀, FI/OD₆₀₀ and induction/repression ratio of *E. coli* BL21(DE3)/pTOL03E, *E. coli* BL21(DE3)/pTOL03F and *E. coli* BL21(DE3)/pDKS-GFPuv

Plasmid/genetic trait	IPTG induction concentration, μM	OD ₆₀₀ ^a	Specific FI, FI/OD ₆₀₀ ^a	Induction/repression ratio
pTOL03E/ upstream + ATCG _{avg} +(-35)+(-10) in rich medium	0	5.1 \pm 0.0	155 \pm 1	285
	200	4.0 \pm 0.1	44 121 \pm 247	
pTOL03F/ (-10) in rich medium	0	5.0 \pm 0.0	333 \pm 10	122
	15	4.0 \pm 0.1	40 597 \pm 446	
pTOL03F/ (-10) in M9 medium	0	2.4 \pm 0.0	98 \pm 2	276
	500	2.1 \pm 0.0	27 196 \pm 176	
pDSK-GFPuv/constitutive GFPuv expression in rich medium	NA	4.0 \pm 0.1	22 878 \pm 3742	NA ^b

^aSamples were collected at 8–8.5 h for pDSK-GFPuv and at 9 h for pTOL03E and pTOL03F in the rich medium. Samples were collected at 12 h for pTOL03F in the minimal medium.

^bThe induction/repression ratio cannot be defined for a constitutive promoter.

**Figure 7.** The dependence of specific FI of (A) pTOL03E and (B) pTOL03F on the AtxA induction, which was driven by IPTG. The supplementation of IPTG was achieved when OD₆₀₀ of bacterial cultures reached 0.4–0.6. Errors represent standard deviation with $n = 3$.

value of $44\,121 \pm 247$ at $200\ \mu\text{M}$ (Figure 7A). Therefore, the induction/repression ratio was calculated as 285 (Table 3). The FI/OD₆₀₀ 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/OD₆₀₀ showed a saturation value of $40\,597 \pm 446$ at $15\ \mu\text{M}$ 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 OD₆₀₀ of 4.0 ± 0.1 for both strains (Table 3). The induction/repression ratio of pTOL03F could be improved from 132 to 276 when cultured in M9 minimal medium supplemented with 20 g/l glucose (Table 3). This increase in the induction/repression ratio in response to the M9 minimal medium was consistent with the results of the P_{BAD} system (3). A higher IPTG concentration ($500\ \mu\text{M}$) was required for pTOL03F in the M9 minimal medium to reach full induction, and this can be attributed to the sterile medium for bacterial growth and protein overexpression. The performance of pTOL03E and pTOL03F as expression vectors was compared with that of *E. coli* BL21(DE3)/pDKS-GFPuv. The plasmid pDKS-GFPuv (30) is known for its high green fluorescent protein (GFP) expression under the control of the promoter, P_{PsbA}, where P_{PsbA} is a constitutive promoter and can be 18-fold more efficient than P_{T7} (31). This study, therefore, confirmed that the expression of GFPuv in BL21(DE3)/pDKS-GFPuv was growth-associated (data not shown) and evidenced that BL21(DE3)/pDKS-GFPuv provided a peak FI/OD₆₀₀ of $22\,878 \pm 3742$ at 8–8.5 h (Table 3). Although

the induction/repression ratio cannot be defined for a constitutive promoter, the basal FI/OD₆₀₀ level of pDKS-GFPuv could still be perceived when the bacterial culture first entered the log phase at 2–2.5 h, where the FI/OD₆₀₀ values were in a range of 1747–6805. The AtxA-regulated transcription had a better performance concerning strength and basal level expression compared to pDKS-GFPuv.

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

To achieve the stationary-phase PHB production, *E. coli* BL21P/pTOL03phaCAB 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 $15\ \mu\text{M}$, respectively. It is shown in Figure 8 that a PHB yield, content and titer of $0.20 \pm 0.27\ \text{g/g-glucose}$, $68 \pm 11\%$ and $1.5 \pm 0.4\ \text{g/l}$ can be obtained at cultivation time of 12 h with the glucose consumption of $7.3 \pm 3.5\ \text{g/l}$ (data not shown). When glucose was completely consumed, a PHB yield, content and titer of $0.19 \pm 0.0\ \text{g/g-glucose}$, $58 \pm 4\%$ and $1.9 \pm 0.3\ \text{g/l}$ 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

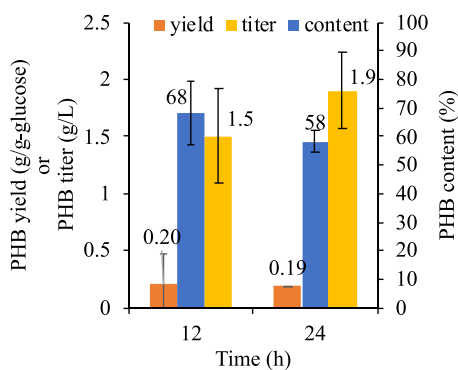


Figure 8. PHB production in *E. coli* BL21P/pTOL03FphaCAB. *E. coli* BL21P/pTOL03FphaCAB was first aerobically cultivated in LB for 6 h, followed by the supplementation of glucose and IPTG to reach the final concentrations of 10 g/l and 15 μ M, respectively. Errors represent standard deviation with $n = 3$.

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 pTOL02F + pTOLAtxA and pTOL03F). 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 SLI/SLII 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 pTOL02B and pTOL03B 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_{T7} (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 $\sim 2.6 \times 10^5$ (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, modifiable 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 vivo* nonspecific binding activity can be found in the examples of λ CI and Cro repressors (44).

Another interesting DNA fragment of 509sequence 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 P_{AtxA} can rule out the dependence of σ^{70} because the −35 sequence is not necessary for pTOL03F. Instead, it is the σ^S that is involved in P_{AtxA} for the following arguments. First, P_{AtxA} is a stationary-specific promoter. Second, the −10 sequence of TATACT used in P_{AtxA} is known as the type III promoter (28) and can be recognized by σ^S . Third, the dependence of recombinant protein induction on high concentration of NaCl indicates the significant role of σ^S (29). The strong activity of P_{AtxA} confirms that sigma factors other than σ^{70} can be used in applications. This is interesting because the intracellular concentrations of σ^S is one order of magnitude lower than that of the σ^{70} at the log phase and is half of that of σ^{70} (46). Moreover, the *in vitro* data have shown that the binding affinity of σ^S to the core RNAP is only half of that of σ^{70} (47). The role of σ^S in P_{AtxA} should be thoroughly investigated, especially σ^S 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 P_{AtxA} 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 40 688 ± 446 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*. P_{AtxA} requires 15 μ M 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 P_{AtxA}, 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 P_{AtxA} 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 P_{AtxA} 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 P_{AtxA} developed in this study.

Supplementary data

Supplementary data are available at SYN BIO 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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