Engineering Microbiology 3 (2023) 100098

Contents lists available at ScienceDirect

Engineering Microbiology

journal homepage: www.elsevier.com/locate/engmic

Original Research Article

Identification of multiple regulatory genes involved in TGase production in *Streptomyces mobaraensis* DSM 40587

Xian Liu[a,b], Dan Wang[a,b], Yuru Zhang[c], Xiaoxin Zhuang[c], Linquan Bai[a,b,*]

[a] State Key Laboratory of Microbial Metabolism, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] Joint International Research Laboratory of Metabolic & Developmental Sciences, Shanghai Jiao Tong University, Shanghai 200240, China
[c] Taixing Dongsheng Bio-Tech Co., Ltd., Taixing 225411, China

ARTICLE INFO

Keywords:
Transglutaminase
Streptomyces mobaraensis
AdpA
Transcription
Regulatory network

ABSTRACT

Microbial transglutaminase (TGase) is a protein that is secreted in a mature form and finds wide applications in meat products, tissue scaffold crosslinking, and textile engineering. *Streptomyces mobaraensis* is the only licensed producer of TGase. However, increasing the production of TGase using metabolic engineering and heterologous expression approaches has encountered challenges in meeting industrial demands. Therefore, it is necessary to identify the regulatory networks involved in TGase biosynthesis to establish a stable and highly efficient TGase cell factory. In this study, we employed a DNA-affinity capture assay and mass spectrometry analysis to discover several transcription factors. Among the candidates, eight were selected and found to impact TGase biosynthesis. Notably, SMDS_4150, an AdpA-family regulator, exhibited a significant influence and was hence named $AdpA_{Sm}$. Through electrophoretic mobility shift assays, we determined that $AdpA_{Sm}$ regulates TGase biosynthesis by directly repressing the transcription of *tg* and indirectly inhibiting the transcription of *SMDS_3961*. The latter gene encodes a LytR-family positive regulator of TGase biosynthesis. Additionally, $AdpA_{Sm}$ exhibited negative regulation of its own transcription. To further enhance TGase production, we combined the overexpression of *SMDS_3961* with the repression of *SMDS_4150*, resulting in a remarkable improvement in TGase titer from 28.67 to 52.0 U/mL, representing an 81.37% increase. This study establishes AdpA as a versatile regulator involved in coordinating enzyme biosynthesis in *Streptomyces* species. Furthermore, we elucidated a cascaded regulatory network governing TGase production.

1. Introduction

The commercial microbial transglutaminase (TGase) is a single-subunit secreted protease with a molecular weight of 37,863 Da and is composed of 331 amino acids. Its crystal structure appears disk-like, with a catalytic active site consisting of Cys64-Asp255-His274 [1]. Following secretion through the Tat pathway outside the cell [2], the N-terminal zymogen of pro-TGase (44 amino acids) is cleaved by TAMEP [3], and the remaining tetrapeptide is removed by a serine protease [4], resulting in the formation of mature TGase. This mature form of TGase catalyzes various reactions such as cross-linking, ligation, and deamidization [1]. TGase has found extensive use in the food industry, where the crosslinks formed by ε-(γ-glutamyl) lysine isopeptide bond (G-L bond) enhance the appearance and taste of TGase-treated foods like ham sausage and rolled meat. Additionally, the integration of amino acids increases the nutritional value of these foods [5,6]. In the pharmaceutical industry, TGase mediates the cross-linking of collagen and gelatin, producing scaffolds for organ regeneration. Moreover, TGase is utilized in the chemo-enzymatic bioconjugation process to obtain antibody-drug conjugates (ADCs) [7–10]. Furthermore, TGase contributes to improving fabric strength and repairing broken fibers in the textile industry [7].

In practical applications, *Streptomyces mobaraensis* stands as the exclusive permissible host for producing food-grade TGase [11]. However, the current production of TGase derived from *Streptomyces mobaraensis* has encountered significant limitations [12]. Attempts at the heterologous expression in *Escherichia coli* and *Corynebacterium glutamicum* necessitate RNA-dependent chaperones for proper folding and proteases for post-modification or risk the formation of inclusion bodies. These challenges lead to difficulties in enzyme activation, separation, and purification [13–16]. Consequently, researchers are actively developing methods for metabolic engineering within the original hosts. Common genetic manipulations employed to increase the titer of secreted enzymes include: (i) enhancing expression or translation effi-

* Corresponding author at: State Key Laboratory of Microbial Metabolism, School of Life Sciences & Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China.
E-mail address: bailq@sjtu.edu.cn (L. Bai).

https://doi.org/10.1016/j.engmic.2023.100098
Received 2 October 2022; Received in revised form 7 June 2023; Accepted 9 June 2023
Available online 10 June 2023

ciency through promoter selection, optimization of codon bias, or plasmid selection [17]; (ii) improving secretory efficiency through signal peptide selection or optimization, overexpression of SPase, or selection and optimization of the Tat/Sec pathway [18–20]; (iii) alleviating metabolic burdens through medium optimization (protease, $MgCl_2$, CTAB, bleomycin sulfate, PMSF, and NaF) [21–24], genome reduction, or bioprocess optimization; (iv) mitigating stress responses by overexpressing proteins such as PspA, RpoS, or sigma factors [25]; (v) employing morphological transformations like SsgA or AdpA overexpression [14,26,27]. In addition to these strategies, random mutagenesis techniques, including UV or NTG mutagenesis and atmospheric and room-temperature plasma (ARTP), are frequently utilized in industrial settings [28,29]. However, to achieve effective rational engineering, it is crucial to identify the regulatory pathways governing TGase expression. To date, LuxR is the only confirmed essential positive regulator that contributes to mycelial growth in *S. mobaraensis* and TGase biosynthesis [23].

The DNA affinity capture assay (referred to as DACA) has proven to be a highly effective method for capturing and discovering transcription factors involved in the regulatory pathways of antibiotic production [30–32]. It is well recognized that morphological differentiation and secondary metabolism in *Streptomyces* are subject to complex and precise regulation by transcription factors, including global regulators, pleiotropic regulators, and pathway-specific regulators [33]. AdpA, a member of the AraC/XylS family, is an A-factor-dependent protein and serves as a global transcription factor in various *Streptomyces* species, influencing the expression of genes involved in secondary metabolism and morphogenesis [31,34–38]. For example, in *Streptomyces griseus*, AdpA acts as a pathway-specific regulator for *strR* and *griR* in streptomycin and grixazone biosynthesis, respectively [34,39]. AdpA also plays a role in controlling the expression of genes such as *adsA*, which encodes a sigma factor of RNA polymerase gene, *sgmA*, a metalloendopeptidase gene, *amfR*, a regulator gene for aerial mycelium forming, and *ssgA*, a gene involved in spore septum formation [40–43]. Furthermore, AdpA regulates trypsin genes (*sprT* and *sprU*), chymotrypsin genes (*sprA, sprB*, and *sprD*), and other members of the AdpA regulon [44,45]. Through chromatin immunoprecipitation (ChIP) with AdpA in *S. griseus*, several putative genes encoding intracellular and extracellular proteases, as well as genes associated with transport and stress responses, which are likely involved in primary metabolism, were identified. Some of these genes were further confirmed using electrophoretic mobility shift assays (EMSA) [46–48]. DNase I footprinting assays revealed a relatively conserved binding site 5′-TGGCSNGWWY-3′ (S: G or C; W: A or T; Y: Tor C; N: any nucleotide) for AdpA. The HTH-1 (helix-turn-helix) domain located in the N-terminus of AdpA recognizes the TGGCS sequence, with the nucleotide C playing a crucial role, while the HTH-2 of the C-terminus of AdpA recognizes the remaining nucleotides [49]. AdpA can bind to two types of binding sites as a dimer: the “long site,” where each monomer of the dimer binds to one site, and the “short site,” where only one monomer of the dimer binds to a site, while the other monomer does not [50].

In this study, we employed 5′-RACE to determine the transcription start site (TSS) and the intergenic region of the TGase gene. This information was utilized for the identification of transcriptional regulatory proteins using the DNA affinity capture assay. Subsequently, coupled tandem mass spectrometry (LC-MS/MS) was employed to detect and characterize these regulatory proteins, elucidating their roles in TGase biosynthesis. Through genetic manipulation targeting these regulators, we achieved significant improvements in TGase production.

2. Materials and methods

2.1. Strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table S1.

S. mobaraensis DSM 40587 and TX (Dongsheng Bio-Tech Co., Ltd., Taixing, China) were grown in ISP4MYM medium (10 g/L soluble starch, 2 g/L $(NH_4)_2SO_4$, 1 g/L K_2HPO_4, 2 g/L $CaCO_3$, 1 g/L NaCl, 1 g/L $MgSO_4$, 100 μL/L inorganic salt solution (1% $ZnSO_4$, 1% $MnCl_2$, 1% $FeSO_4$), 1 g/L mannitol, 1 g/L yeast extract, 1 g/L malt extract, 20 g/L agar) for 7 days to allow sporulation. Conjugation of *Streptomyces* with *E. coli* was carried out on ISP4MYM plates supplemented with 10 mM $MgCl_2$. For fermentation, *S. mobaraensis* DSM 40587, TX, and their derivatives were grown in 30 mL of seed medium (20 g/L glycerol, 6 g/L yeast extract, 25 g/L fish meal peptone, 2 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $K_2HPO_4{\cdot}3H_2O$, at a pH of 7.4). Subsequently, 3 mL of the seed culture was transferred into 30 mL of fermentation medium (20 g/L glycerol, 6 g/L yeast extract, 25 g/L fish meal peptone, 2 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $K_2HPO_4{\cdot}3H_2O$, pH 7.4) and cultured for 30 h with shaking at 220 rpm at 30 °C.

E. coli ET12567(pUZ8002) was used for conjugation. The *E. coli* cells were cultured in Luria-Bertani (LB) broth at 37 °C.

2.2. DNA affinity capture assay (DACA)

The mycelia of DSM 40587 grown in fermentation medium for 30 h were harvested and resuspended in 100 mL of binding buffer (20 mM Tris–HCl, pH 8.0, 50 mM NaCl, 10% glycerol, 1 mM EDTA), and broken using an ultra-high-pressure crusher (SCIENTZ, Zhejiang, China) (40% power, work 3 s, stop 3 s, 5 min/cycle for 3 cycles), followed by centrifugation (12,000 rpm for 25 min at 4 ˚C) to obtain the supernatant. The supernatant was filtered through a 3 kDa Millipore filter, and the protein concentration was detected using a Nanodrop.

Biotinylated P*tg* probes were amplified using the genomic DNA of DSM 40587 and plasmid with biotin-labeled primers P*tg*-F/R. Probes (10 μg) were individually added to 1 mL of streptavidin-agarose (SA) (GE, Boston, MA USA) and incubated in the binding buffer at room temperature for 30 min. The biotinylated P*tg* probes coupled with SA were incubated with 150 mg of total protein extracted from the mycelia of DSM 40587, supplemented with sonicated salmon sperm DNA (Solarbio, Beijing, China) for 60 min at 4 ˚C, followed by centrifugation (12,000 rpm for 2 min at 4 ˚C) two to three times to remove nonspecifically bound proteins. The bound proteins were eluted using elution buffer (20 mM Tris–HCl, pH 6.8, 1 M NaCl, 10% glycerol, 1 mM EDTA) at room temperature for 20 min, centrifuged (12,000 rpm for 2 min), isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Protein Stains O silver staining (Sangon Biotech Co., Ltd., Shanghai, China), and identified through liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a system consisting of the Easy-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA USA) and Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). The obtained data were analyzed against the UniProt database of *S. mobaraensis* DSM 40587 and genomic information [32].

2.3. Construction of plasmids for the overexpression and knock-down of regulator genes

The eight regulator genes were obtained through PCR amplification, followed by ligation into *Eco*RV-digested pBluescript SK vectors. The resulting constructs were sequenced for confirmation. Subsequently, these plasmids were digested with *Eco*RI and *Nde*I, and the fragments containing the regulator genes were ligated into an *Eco*RI/*Nde*I-digested plasmid called pLQ646. The primers used for gene overexpression can be found in Table S2.

To achieve gene knock-down using CRISPRi/dCas9, an N20 sequence for each gene was designed using the website crispy.secondarymetabolites.org. The sgRNA scaffolds were obtained through PCR amplification and assembled into *Spe*I/*Eco*RI-digested pSET-dCas9 vectors. The primers used for gene knock-down can be found in Table S2.

2.4. Conjugation between streptomyces mobaraensis and E. coli

To introduce the plasmids, a stepwise approach was followed in both the non-methylating *E. coli* strain ET12567(pUZ8002) and *S. mobaraensis* strains. Spores (~10^9) were subjected to a heat treatment at 50 °C for 10 min and pregerminated for 2 h. The pregerminated spores were then mixed with *E. coli* cells. The resulting suspensions were spread onto non-selective plates containing ISP4MYM medium supplemented with 20 mM $MgCl_2$. After an incubation period of 16 h at 30 °C, antibiotics were overlaid onto the plates. Typically, exconjugants appeared after 3 days [51].

For the verification of gene overexpression, the exconjugants were subjected to PCR using the primers *kasOp*-YZ-F and *smds_X*-YZ-R (Table S2). In contrast, for gene knock-down, the exconjugants were verified using PCR with the primers dCas9-YZ-F/R (Table S2).

2.5. Enzyme activity analysis of TGase

The fermentation broth was subjected to centrifugation, and 200 μL of the resulting supernatant was taken and diluted. It was then mixed with 2 mL of preheated reagent A (0.2 M Tris–HCl, 0.1 M hydroxylamine hydrochloride, 0.01 M GSH, 0.03 M Na-CBZ-GLN-GLY, pH 6.0) and incubated in a water bath at 37 °C for 10 min. To stop the reaction, 2 mL of reagent B (composed of an equal volume of 3 mol/L HCl, 12% TCA, and 5% $FeCl_3$ in 0.1 mol/L HCl) was added. The OD_{525} was measured using a spectrophotometer [52]. Enzyme activity = [(9.1965×OD_{525}−0.055)/2*dilution ratio].

2.6. RNA extraction

For RNA extraction, fermentation samples were collected and subjected to centrifugation. The resulting precipitated mycelia were resuspended in 1 mL of Redzol reagent (SBS Genetech, Shanghai, China) and disrupted for 1 min. Then, 200 μL of phenol-chloroform was added to the samples, followed by vortexing and centrifugation at 4 °C for 10 min. The liquid layer was carefully transferred to a new tube, and 200 μL of chloroform was added. The mixture was centrifuged again. The RNA pellets were washed with ethanol and washing buffer and then dissolved in DEPC water. The concentration and purity of RNA were determined using a Nanodrop. To remove any contaminated DNA, DNase I treatment was performed using DNase I (Thermo Fisher Scientific, Waltham, MA USA).

2.7. Quantitative real-time PCR

Mycelia of *S. mobaraensis* DSM 40587 were harvested, and total RNA was extracted following the manufacturer's instructions (SBS Genetech, Shanghai, China). The quality of the RNA was assessed using a Nanodrop 2000 spectrophotometer. For qPCR experiments, the total RNA was reverse transcribed into cDNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA USA). qPCR experiments were performed on a 7500 Fast Real-time CR system (Applied Biosystems, Waltham, MA USA) using the Maxima™ TB Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA) following the manufacturer's protocol. The expression levels of the target genes were calculated using the $2^{-\Delta\Delta CT}$ method, with the housekeeping gene *hrdB* serving as the internal control [53].

2.8. Electrophoretic mobility shift assays (EMSA)

DNA probes were amplified by PCR using the primers listed in Table S2. The amplified probes were then mixed independently with purified recombinant proteins in the binding buffer, along with unlabeled DNA, 10×poly(dI·dC), and ddH_2O, resulting in a 20 μL reaction mixture. The mixture was incubated at 25 °C for 30 min. After incubation, the reaction mixtures were separated on 6% native PAGE gels using 0.5×Tris-acetate-EDTA buffer as the running buffer at 100 V for 60 min. The gels were subsequently scanned for fluorescence using a Cy2 Fltr 525BP20 filter on an Amersham Typhoon RGB scanner (GE, Boston, MA USA) [54].

2.9. DNase I footprinting

DNA probes were amplified by PCR using the primers listed in Table S2. The amplified probes were then independently mixed with purified recombinant proteins in the binding buffer, unlabeled DNA, and ddH_2O, resulting in a 20 μL reaction mixture. The mixture was incubated at 25 °C for 30 min. Subsequently, 10 μL of a solution with 1 unit of DNase I (Thermo Fisher Scientific, Waltham, MA USA) and 100 nM $CaCl_2$ was added, and the mixture was digested for 1 min at 37 °C. To stop the digestion, 140 μL of DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM EDTA, 0.15% sodium dodecyl sulfate) was added. The digested product was then mixed with 10 μL of phenol-chloroform and centrifuged to remove proteins, and the DNA was extracted through ethanol precipitation. The resulting DNA was dissolved in 30 μL of Mili-Q water and sequenced using an ABI sequencer (Applied Biosystems, Waltham, MA USA). The molecular weight standard used was ABI GeneScan500 Liz [32].

2.10. Genome sequence deposition

The genome sequence of *S. mobaraensis* DSM 40-587 was deposited into Genbank with an accession number of S. GCA_020099395.1.

3. Results

3.1. Identification of eight candidate regulatory proteins capable of binding to the tg promoter

To identify the promoter region of the TGase gene (*tg*), the transcription start site (TSS) of *tg* was determined using 5′-RACE, revealing its location 415 bp upstream of the TGase open reading frame (ORF). The predicted −10 and −35 regions for RNA polymerase binding were also identified (Fig. 1A). Subsequently, a 452-bp biotinylated probe, named P*tg*, was designed to cover the intergenic region from the stop codon of *SMDS_1473* to the TSS. This probe was used in the DNA affinity capture assay (DACA) to isolate regulatory proteins from total proteins of *Streptomyces mobaraensis* DSM 40587 (hereafter referred to as DSM 40587). The assay involved two or three rounds of washing (Fig.1B, S1, S2, S3). Through LC-MS/MS analysis, a total of 76 transcription factors (TFs), 31 DNA binding proteins, and 4 sigma factors were identified in the twice-washed sample. In the three-times-washed sample, 61 TFs, 43 DNA binding proteins, and 5 sigma factors were identified. Notably, eight TFs, namely SMDS_1792, SMDS_1797, SMDS_4150, SMDS_720, SMDS_4036, SMDS_3072, SMDS_2341, and SMDS_3961 displayed higher scores in both samples (Table 1, S3, S4). According to the genome annotation of DSM 40587 [28], SMDS_4150 was annotated as an AdpA-family regulator, sharing 84.69% DNA homology with *adpA* in *Streptomyces griseus*. Among the eight TFs, SMDS_1792, SMDS_1797, SMDS_720, SMDS_3072, and SMDS_3961 all belonged to two-component regulatory systems (TCSs). SMDS_1792 and SMDS_1797 possessed histidine kinase domains (HK), while SMDS_3961 contained the response regulator domain (RR) of the LytS/R family. Interestingly, no HK gene was found near *smds_3961* in the genome, suggesting that SMDS_3961 may function as an orphan TCS. SMDS_720 and SMDS_3072 exhibited both HK and RR domains. SMDS_2341 was identified as an AfsR-family global transcriptional regulator [55], and SMDS_4036 belonged to the HU_IHF family (Fig.S4).

3.2. Six regulatory genes contributing to TGase biosynthesis

To investigate the involvement of these regulators in TGase biosynthesis, each gene was overexpressed in *S. mobaraensis* TX, an indus-

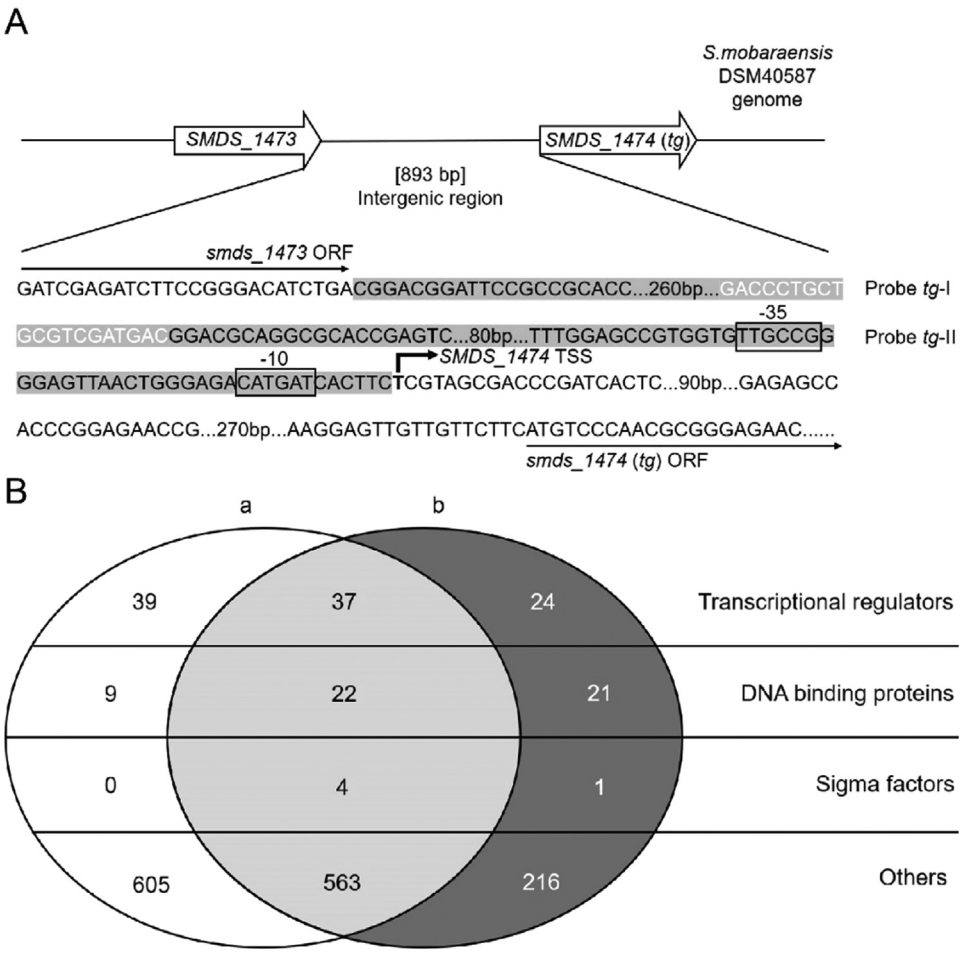

Fig 1. A. Transcription unit of *tg*. The transcription start site is indicated by a bold arrow, and the −10 and −35 regions are enclosed in boxes. Probes *tg*-I and *tg*-II for EMSA are shaded in gray, and their overlapping sequences are shown in white. B. Number of proteins captured by DACA. Ellipse a represents proteins captured after two washes to remove nonspecific binding proteins, while ellipse b represents proteins captured after three washes. The 626 proteins shared by both ellipses are highlighted with a light gray background.

Table 1
Candidate regulatory proteins identified by DACA.

Regulator	Ion score (−10lgP)	Gene product
SMDS_4150	228.71	AdpA-family regulator
SMDS_1792	207.32	NIT/BaeS/ICP4-family hybrid two-component system (HK)
SMDS_4036	203.08	HU_IHF-family DNA binding protein
SMDS_3072	197.91	AtoS/BaeS/CheY-family hybrid two-component system (HK & RR)
SMDS_2341	184.79	AfsK/R-family two-component system (RR)
SMDS_1797	171.64	NIT/BaeS-family hybrid two-component system (HK)
SMDS_720	154.17	MCP/GAF/BarA/CheY-family hybrid two-component system (HK & RR)
SMDS_3961	147.84	LytS/R-family two-component system (RR)

trially high-yielding strain derived from DSM 40587. The TGase production and *tg* transcription were analyzed by enzymatic reaction and real-time quantitative PCR (qPCR) in each mutant strain. Compared to the control strain TX::pLQ646 carrying the vector plasmid pLQ646 integrated into the chromosome, overexpression of *SMDS_4036, SMDS_2341, SMDS_1797*, and *SMDS_3961* resulted in a 7.09% to 69.76% increase in TGase production, while overexpression of *SMDS_4150, SMDS_1792, SMDS_3072*, and *SMDS_720* led to a 17.29% to 60.34% decrease in TGase production (Fig. 2A). Moreover, qPCR analysis revealed that the transcription of *tg* was significantly enhanced by the overexpression of *SMDS_4036, SMDS_2341, SMDS_1797*, and *SMDS_3961* and suppressed by the overexpression of *SMDS_4150* and *SMDS_720* (Fig. 2B). These findings were consistent with the changes observed in TGase production. Therefore, *SMDS_4036, SMDS_2341, SMDS_1797*, and *SMDS_3961* are considered positive regulatory genes for *tg* expression, with *SMDS_3961*, a member of the LytS/R-family, exhibiting the highest effectiveness. On the other hand, *SMDS_4150* and *SMDS_720* are regarded as negative regulatory genes, with *SMDS_4150*, an AdpA-family gene, being more influential. Consequently, SMDS_3961 and SMDS_4150 were selected for further mechanistic characterization.

3.3. *LytS/R-family SMDS_3961 directly upregulated transcription of* tg

Electrophoretic mobility shift assays (EMSAs) and DNase I footprinting were conducted to investigate the regulatory mechanism of SMDS_3961 on P*tg*, the promoter of *tg*. Since the 452-bp region upstream of the TSS was designated as P*tg*, two probes P*tg*-I and P*tg*-II were designed with a 20-bp overlapping sequence to cover the entire P*tg* region (Fig. 1A). A full-length SMDS_3961 protein was utilized for the DNA-protein binding assays (Fig.S5). The results revealed that SMDS_3961 was specifically bound to P*tg*-I, while no binding was observed with P*tg*-II (Fig. 3A-B). In many studied systems, response regulators containing the LytTR domain typically form dimers and bind to consensus sequences such as the LytR box 5′-CCCAGTTNTNCAC-3′ or 9-bp direct repeats 5′-ACAGTTAAG-3′, separated by 12 bp and located just upstream of the TSS [56,57]. DNase I footprinting analysis indicated that the bind-

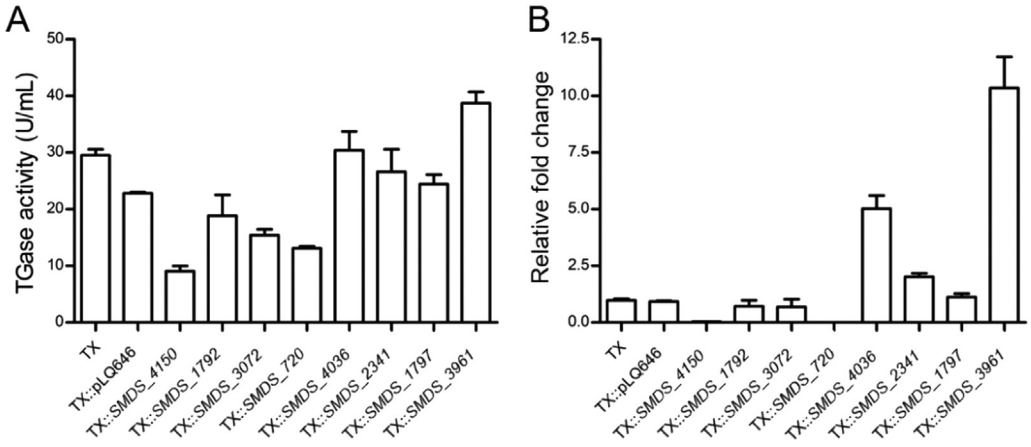

Fig 2. TGase activity (A) and transcription of *tg* (B) across eight mutants with each regulatory gene overexpressed.

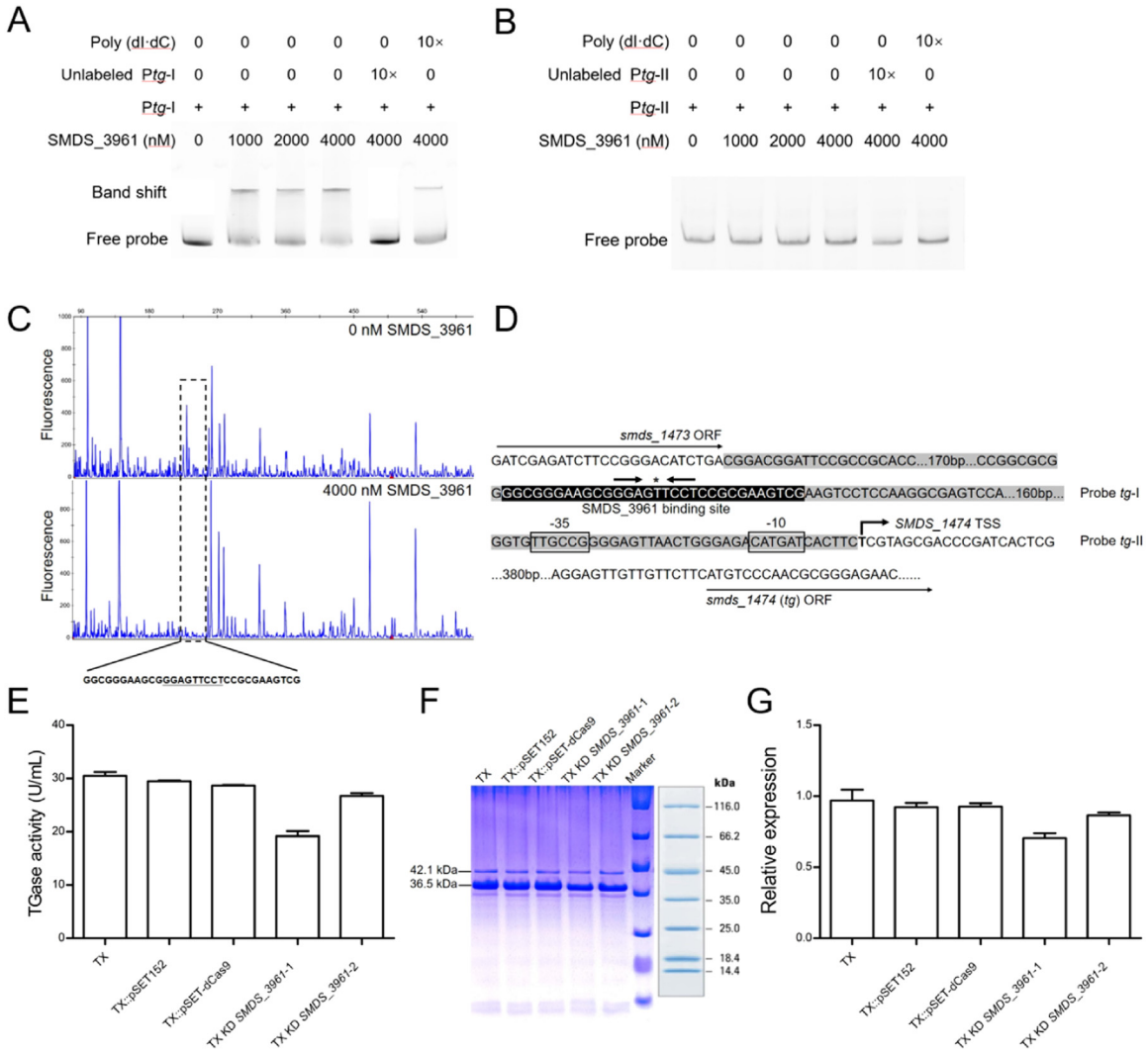

Fig 3. SMDS_3961 is involved in TGase biosynthesis and binds to P*tg*. A. EMSA showing the interaction between SMDS_3961 and P*tg*-I probe. B. EMSA demonstrating the absence of binding between SMDS_3961 and P*tg*-II probe. C. DNase I footprinting assays using varying amounts of SMDS_3961 protein. D. Sequence of the SMDS_3961-binding site indicated with a black background. The conserved consensus sequence is marked with an asterisk and arrows. E. TGase activity in *S. mobaraensis* TX and *SMDS_3961* knock-down mutants. F. SDS-PAGE analysis of TGase expression in *SMDS_3961* knock-down mutants and controls. The 30-hour fermentation broth of TX, TX::pSET152, TX::pSET-dCas9, TX KD *SMDS_3961*–1&2 was collected, centrifuged, and equal volumes of supernatant were analyzed using SDS-PAGE. Bands at 36.5 kDa represent mature TGase, and bands at 42.1 kDa represent pro-TGase. G. Transcriptional activity of *tg* in *S. mobaraensis* TX and *SMDS_3961* knock-down mutants.

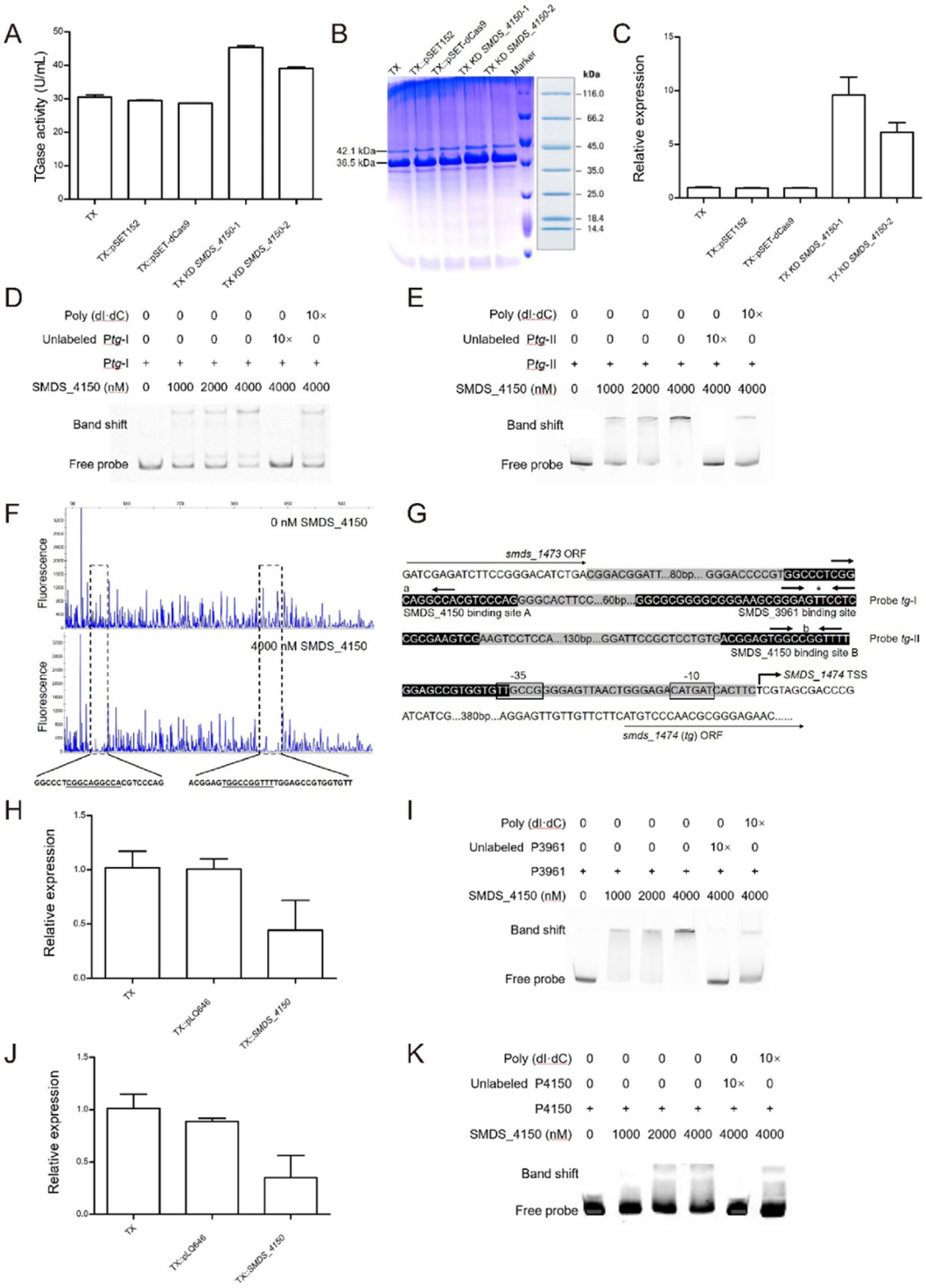

Fig 4. The AdpA-family regulator SMDS_4150 was found to suppress the transcription of *tg*, *SMDS_3961*, and its own gene through direct binding. A. The TGase production of TX and the *SMDS_4150* knock-down mutants. B. SDS-PAGE analysis of TGase expression in the *SMDS_4150* knock-down mutants and controls. The 30-hour fermentation broth of TX, TX::pSET152, TX::pSET-dCas9, TX KD *SMDS_4150*–1&2 was collected and centrifuged, and equal volumes of supernatant were taken and analyzed using SDS-PAGE. The 36.5 kDa bands represented mature TGase, and the 42.1 kDa bands represented pro-TGase. C. Transcription of *tg* in TX and the *SMDS_4150* knock-down mutants. D. EMSAs with SMDS_4150 and the P*tg*-I probe. E. EMSAs with SMDS_4150 and the P*tg*-II probe. F. DNase I footprinting using various amounts of SMDS_4150 protein bound to the P*tg* probe. G. SMDS_4150 binding sites A or B are indicated with a black background. The conserved AdpA consensus sequences are marked with a or b and arrows. H. Transcription of *SMDS_3961* in TX and the TX::*SMDS_4150* mutant. I. EMSA with SMDS_4150 and the P3961 probe. J. Transcription of *SMDS_4150* in TX and the TX::*SMDS_4150* mutant. K. EMSA with SMDS_4150 and the P4150 probe.

ing site of SMDS_3961 was positioned upstream of the −35 region of P*tg* and exhibited moderate similarity to the consensus binding sequence, with only one repeat present (Fig. 3CD).

To further explore the role of *SMDS_3961*, knock-down mutants were generated in *S. mobaraensis* TX using CRISPRi/dCas9 technology. Two different sgRNAs were selected to target distinct locations, with one targeting the upstream region of the *SMDS_3961* ORF (gRNA-1) and another within the *SMDS_3961* ORF (gRNA-2). Enzymatic reaction, SDS-PAGE, and qPCR analysis revealed that TGase production decreased by 33.1% (gRNA-1) and 6.8% (gRNA-2) (Fig. 3E-F), and the transcription of *tg* was significantly suppressed (Fig. 3G). Collectively, these findings confirm the positive involvement of SMDS_3961 in TGase biosynthesis through direct binding to the P*tg* promoter.

3.4. AdpA-family member SMDS_4150 suppressed the transcription of tg, SMDS_3961, and its own gene

The previous overexpression experiments indicated that SMDS_4150, an AdpA-family regulator, has a negative impact on TGase production. To further confirm its function, *SMDS_4150* knock-down mutants were generated using gRNA-1 to target a site upstream of the *SMDS_4150* ORF and gRNA-2 to target a site within the *SMDS_4150* ORF. Both mutants exhibited increased TGase production by 58.0% (gRNA-1) or 36.4% (gRNA-2) (Fig. 4A-B), and the transcription of *tg* was significantly reduced in the SMDS_4150 knock-down mutants (Fig. 4C). EMSA and DNase I footprinting assays revealed that SMDS_4150 bound to different regions within both P*tg*-I and P*tg*-II (Fig. 4DE), where the highly conserved AdpA consensus sequence 5′-TGGCSNGWWY-3′ (S: G or C; W: A or T; Y: T or C; N: any nucleotide) was located [49] (Fig. 4FG).

Interestingly, when analyzing the transcription of *SMDS_3961* in the TX::*SMDS_4150* mutant, which overexpressed *SMDS_4150*, its expression was found to be reduced by 55.7% (Fig. 4H), suggesting a negative regulatory role of SMDS_4150. Sequence analysis of the promoter region of *SMDS_3961* (P3961) identified a conserved binding site for AdpA, 5′-TGGCSNGWWY-3′. Using P3961 as a probe, SMDS_4150 exhibited specific binding to this region (Fig. 4I). Moreover, the transcription of *SMDS_4150* in the TX::*SMDS_4150* mutant was measured and found to be severely reduced as expected (Fig. 4J), and EMSA demonstrated the binding of SMDS_4150 to its own promoter region P4150 (Fig. 4K). In other words, AdpA regulated TGase metabolism in a pleiotropic manner, both directly and indirectly. Since the global transcription factor AdpA affects morphogenesis in many *Streptomyces* species, the growth condition of the *SMDS_4150* knock-down mutant was also examined, revealing its negative involvement in aerial hyphae formation (Fig. S6).

3.5. Combined overexpression and knock-down of regulatory genes enhance the production of TGase

The results mentioned above suggest that a tandem overexpression of positive TFs or knock-down of negative TFs can be an effective strategy to enhance TGase production. In light of this, the positive regulator *SMDS_3961* was overexpressed, while the negative regulator *SMDS_4150* was suppressed in the same strain. As depicted in Fig. 5, the resulting strain LX59 exhibited an enzyme activity of 52.0 U/mL after 30 h of fermentation, which represents an 81.2% increase compared to the control strain TX::pSET-dCas9 and a 14.7% increase compared to strain TX KD *SMDS_4150*, where the transcription of *SMDS_4150* was suppressed.

4. Discussion

The understanding of the regulatory network governing TGase biosynthesis is still limited. One effective strategy to capture and identify potential TFs is DACA [30–32]. Through this approach, we identified a minimum of 109 potential TFs that may interact with P*tg*. Surprisingly, LuxR (SMDS_3275), the only previously reported TF associated with TGase biosynthesis [23], appeared with low scores in our study.

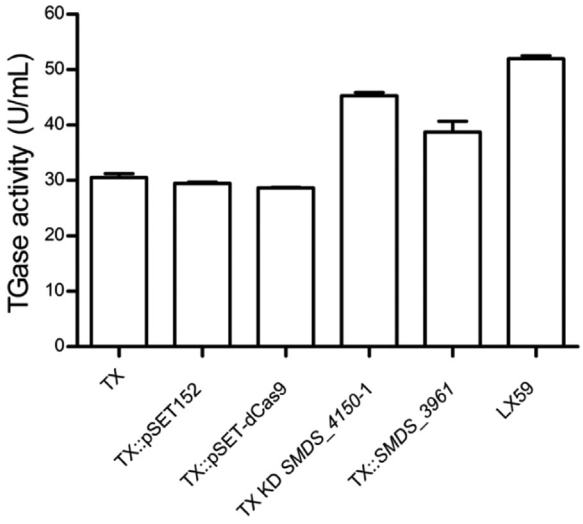

Fig 5. TGase activity of tandem overexpression and knock-down mutants. LX59: TX KD *SMDS_4150*–1::*SMDS_3961*.

However, we discovered that SMDS_4150, the top-ranked AdpA-family regulator, played crucial roles in TGase biosynthesis. It was found to regulate *tg*, $lytR_{Sm}$, and *adpA*, all of which were members of its regulon. SMDS_4150 acted as a repressor for the first two genes and demonstrated autorepression. It is well known that the −10 and −35 regions are critical binding sites for RNA polymerase. If the binding sites of a regulator overlap with these regions or are located between them, it can impede or promote the binding of RNA polymerase. In the case of SMDS_4150, one of its binding sites covered part of the −35 region, which explains its role as a repressor of *tg*. AdpA is a pleiotropic transcriptional regulator and a well-studied member of the AraC/XylS family. It plays a role in coordinating morphological differentiation and regulating secondary metabolism in various *Streptomyces* species [31,34–38]. For instance, in *S. griseus*, it influences morphogenesis by promoting the formation of secreted serine protease and regulates the biosynthesis of streptomycin, polyketide compounds, and grixazone by enhancing the expression of pathway-specific transcriptional activators such as StrR and GirR [40–43]. In *S. xiamenensis* 318, it affects morphogenesis and coordinates the biosynthesis of xiamenmycin and PTMs bidirectionally [58]. However, the role of AdpA in regulating primary metabolism, particularly enzyme expression, has rarely been reported. Although several putative genes encoding intracellular and extracellular proteases, as well as genes involved in transport and stress responses associated with primary metabolism, have been identified in *S. griseus*, their regulatory pathways have not been fully elucidated [46–48]. In this study, we investigated the regulatory roles of SMDS_4150, an AdpA homologue in *Streptomyces mobaraensis* DSM 40587, and discovered its negative coordination of TGase biosynthesis through cascade pathways. These findings provide further evidence of the versatile functions of AdpA in primary metabolism in *Streptomyces*, expanding our understanding of the functions of global regulators. Moreover, they pave the way for future efforts in improving the titer of TGase.

Furthermore, our EMSA analysis indicated that SMDS_3961 played a pivotal role as a coordinator in the TGase regulation pathway. This LytR-family response regulator demonstrated binding activity to P*tg*. Interestingly, the protected region was found upstream of the −35 region, suggesting its role in promoting the recruitment of RNA polymerase. Additionally, the protected region was situated between two “short sites” with the sequence 5′-TGGCSNGWWY-3′, which may hinder AdpA from accessing the promoter and eliminate transcriptional inhibition. Thus, LytR acted as an activator in this context. Hydropathy analysis of the LytS amino acid sequence revealed the presence of six potential transmembrane domains [59]. Unlike many sensor proteins that typically

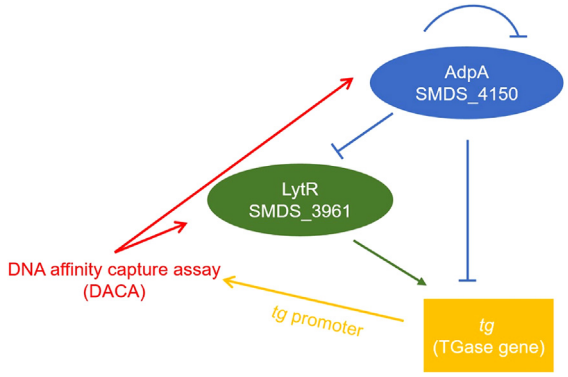

Fig 6. The cascaded regulatory network of TGase based on DACA. The yellow arrow indicates that DACA was performed using the *tg* promoter as a reference. The red arrows represent the identification of two transcriptional regulators, SMDS_4150 and SMDS_3961, through DACA. The green solid arrow signifies that SMDS_3961 activates the transcription of *tg*. The blue lines with termination indicate that SMDS_4150 represses the transcription of *tg*, *SMDS_3961*, and its own gene.

possess two transmembrane domains [60], these multiple transmembrane domains in LytS are well-suited to sense membrane-associated signals such as membrane potential. The response regulator, LytR, belongs to the novel family of non-helix-turn-helix DNA-binding domain proteins. Upon a decrease in membrane potential, LytS undergoes autophosphorylation, subsequently phosphorylating LytR, enabling it to bind to the promoter region of the regulated gene and influence its transcription [61]. Although SMDS_3961 exhibited only 28.68% and 36.00% protein homology with LytR in *Staphylococcus aureus* and *Desulfovibrio vulgaris*, respectively, they shared a typical LytTR domain. The LytTR is a DNA-binding domain of approximately 100 amino acids, belonging to the AlgR/AgrA/LytR family of bacterial transcriptional regulators [62]. It was named after the response regulators LytT in *Bacillus subtilis* and LytR in *Staphylococcus aureus*, which are involved in the regulation of cell autolysis [62]. However, unlike other LytR family regulators, SMDS_3961 did not possess a REC domain, which typically contains a phosphoacceptor site phosphorylated by histidine kinase homologues. This may explain why the associated histidine kinase for this regulator remains unclear, as it is neither located in the gene's vicinity nor identified elsewhere in the genome. This observation highlights the diversity of LytR family regulators and their distinct signal transduction pathways across different bacteria species.

Based on these findings, we propose a cascaded regulatory model in which SMDS_4150 and SMDS_3961 act as core regulators to coordinate TGase production (Fig. 6). This model provides a fresh mechanistic insight into how SMDS_4150 assumes a role similar to that of LytS, thereby forming a novel regulatory pathway involving LytR. However, it remains unknown whether other significant nodes are involved in this regulatory network. Further exploration of TFs with relatively high scores, such as SMDS_16, SMDS_1970, SMDS_864, and others identified in the twice-washed protein eluate, is required to uncover additional players in the system.

5. Conclusion

In this study, we have successfully demonstrated the crucial involvement of AdpA in the regulation of TGase, a secreted enzyme associated with primary metabolism. Furthermore, we have uncovered the unique regulatory pathway of LytR, shedding light on the diverse functions of these transcription factors and enhancing our comprehension of regulatory networks in actinomycetes. In summary, our findings provide substantial evidence for the roles of AdpA and LytR in coordinating TGase biosynthesis, opening up possibilities for the development of more cost-effective strategies for TGase production.

Author Contributions

Xian Liu: Investigation, Methodology, Visualization, Formal analysis, Writing – original draft. Dan Wang: Investigation, Methodology. Yuru Zhang: Investigation, Methodology. Xiaoxin Zhuang: Investigation, Methodology. Linquan Bai: Supervision, Funding acquisition, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given his role as Editorial Board Member, Dr. Linquan Bai had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Dr. Eung-Soo Kim and Dr. Shengying Li.

Acknowledgments

This work was financially supported by the National Key R&D Program of China (2021YFC2100600, 2019YFA0905400), the National Natural Science Foundation of China (31830104), and Science and Technology Commission of Shanghai Municipality (19430750600, 19JC1413000, 17JC1403600). We would like to thank Weishan Wang from the Institute of Microbiology, Chinese Academy of Sciences (CAS), for providing the kasOp* promoter and Taixing Dongsheng Bio-Tech Co., Ltd. for providing the *Streptomyces mobaraensis* DSM 40587 and TX strains.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2023.100098.

References

[1] K. Yokoyama, N. Nio, Y. Kikuchi, Properties and applications of microbial transglutaminase, Appl. Microbiol. Biotechnol. 64 (2004) 447–454.
[2] L. Duarte, C.R. Matte, C.V. Bizarro, M.A.Z. Ayub, Transglutaminases: part I—Origins, sources, and biotechnological characteristics, World J. Microbiol. Biotechnol. 36 (2020) 1–18.
[3] J. Zotzel, R. Pasternack, C. Pelzer, D. Ziegert, M. Mainusch, H.L. Fuchsbauer, Activated transglutaminase from *Streptomyces mobaraensis* is processed by a tripeptidyl aminopeptidase in the final step, Eur. J. Biochem. 270 (2003) 4149–4155.
[4] J. Zotzel, P. Keller, H.L. Fuchsbauer, Transglutaminase from *Streptomyces mobaraensis* is activated by an endogenous metalloprotease, Eur. J. Biochem. 270 (2003) 3214–3222.
[5] S.W. Fatima, S.K. Khare, Current insight and futuristic vistas of microbial transglutaminase in nutraceutical industry, Microbiol. Res. 215 (2018) 7–14.
[6] D. Santhi, A. Kalaikannan, P. Malairaj, S. Arun Prabhu, Application of microbial transglutaminase in meat foods: a review, Crit. Rev. Food Sci. Nutr. 57 (2017) 2071–2076.
[7] L. Duarte, C.R. Matte, C.V. Bizarro, M.A.Z. Ayub, Review transglutaminases: part II—Industrial applications in food, biotechnology, textiles and leather products, World J. Microbiol. Biotechnol. 36 (2020) 1–20.
[8] N. Doti, A. Caporale, A. Monti, A. Sandomenico, F. Selis, M. Ruvo, A recent update on the use of microbial transglutaminase for the generation of biotherapeutics, World J. Microbiol. Biotechnol. 36 (2020) 1–14.
[9] D. Stephan, D. Lukas, K. Roland, K. Harald, R. Nicolas, Site-specific antibody–drug conjugation using microbial transglutaminase, Methods Mol. Biol. 2012 (2019) 135–149.
[10] W. Steffen, F.C. Ko, J. Patel, V. Lyamichev, T.J. Albert, J. Benz, et al., Discovery of a microbial transglutaminase enabling highly site-specific labeling of proteins, J. Biol. Chem. 292 (2017) 15622–15635.
[11] L. Fu, Y. Wang, J. Ju, L. Cheng, Y. Xu, B. Yu, et al., Extracellular production of active–form *Streptomyces mobaraensis* transglutaminase in *Bacillus subtilis*, Appl. Microbiol. Biotechnol. 104 (2020) 623–631.
[12] H.L. Fuchsbauer, Approaching transglutaminase from *Streptomyces* bacteria over three decades, FEBS J. 16060 (2021) 1–24.
[13] J. Lee, A. Son, P. Kim, K.S. Bin, J.E. Yu, G. Han, et al., RNA-dependent chaperone (chaperna) as an engineered pro-region for the folding of recombinant microbial transglutaminase, Biotechnol. Bioeng. 116 (2019) 490–502.
[14] K. Chen, D. Zhang, S. Liu, N.S. Wang, M. Wang, G. Du, et al., Improvement of transglutaminase production by extending differentiation phase of *Streptomyces hygroscopicus*: mechanism and application, Appl. Microbiol. Biotechnol. 97 (2013) 7711–7719.

[15] G. Javitt, Z. Ben-Barak-Zelas, M. Jerabek-Willemsen, A. Fishman, Constitutive expression of active microbial transglutaminase in *Escherichia coli* and comparative characterization to a known variant, BMC Biotechnol. 17 (2017) 1–10.

[16] Y. Kikuchi, M. Date, Y. Kichi, Y. Umezawa, H. Matsui, Secretion of active-form *Streptoverticillium mobaraense* transglutaminase by *Corynebacterium glutamicum*: processing of the pro-transglutaminase by a cosecreted subtilisin-like protease from *Streptomyces albogriseolus*, Appl. Environ. Microbiol. 69 (2003) 358–366.

[17] L. Sevillano, E. Vijgenboom, G.P. van Wezel, M. Díaz, R.I. Santamaría, New approaches to achieve high level enzyme production in *Streptomyces lividans*, Microb. Cell Fact. 15 (2016) 1–10.

[18] J.R. Valverde, S. Gullón, R.P. Mellado, Modelling the metabolism of protein secretion through the Tat route in *Streptomyces lividans*, BMC Microbiol. 18 (2018) 1–11.

[19] Y. Kikuchi, H. Itaya, M. Date, K. Matsui, L.F. Wu, TatABC overexpression improves *Corynebacterium glutamicum* Tat-dependent protein secretion, Appl. Environ. Microbiol. 75 (2009) 603–607.

[20] S. De Keersmaeker, K. Vrancken, L. Van Mellaert, E. Lammertyn, J. Anné, N. Geukens, Evaluation of TatABC overproduction on Tat- and Sec-dependent protein secretion in *Streptomyces lividans*, Arch. Microbiol. 186 (2006) 507–512.

[21] S.W. Fatima, S.K. Khare, Effect of key regulators in augmenting transcriptional expression of transglutaminase in *Streptomyces mobaraensis*, Bioresour. Technol. 340 (2021) 125627.

[22] L. Zhang, L. Zhang, X. Han, M. Du, Y. Zhang, Z. Feng, et al., Enhancement of transglutaminase production in *Streptomyces mobaraensis* as achieved by treatment with excessive $MgCl_2$, Appl. Microbiol. Biotechnol. 93 (2012) 2335–2343.

[23] L. Zhang, L. Sun, H. Yi, S. Wang, J. Han, N. Liu, et al., Comparative proteome analysis of *Streptomyces mobaraensis* under $MgCl_2$ stress shows proteins modulating differentiation and transglutaminase biosynthesis, Food Res. Int. 121 (2019) 622–632.

[24] S. Noda, T. Miyazaki, T. Tanaka, C. Ogino, A. Kondo, Production of *Streptoverticillium cinnamoneum* transglutaminase and cinnamic acid by recombinant *Streptomyces lividans* cultured on biomass-derived carbon sources, Bioresour. Technol. 104 (2012) 648–651.

[25] K. Vrancken, S. De Keersmaeker, N. Geukens, E. Lammertyn, J. Anné, L. Van Mellaert, *pspA* overexpression in *Streptomyces lividans* improves both Sec- and Tat-dependent protein secretion, Appl. Microbiol. Biotechnol. 73 (2007) 1150–1157.

[26] J.M. Willey, A.A. Gaskell, Morphogenetic signaling molecules of the streptomycetes, Chem. Rev. 111 (2011) 174–187.

[27] G.P. Van Wezel, P. Krabben, B.A. Traag, B.J.F. Keijser, R. Kerste, E. Vijgenboom, et al., Unlocking *Streptomyces* spp. for use as sustainable industrial production platforms by morphological engineering, Appl. Environ. Microbiol. 72 (2006) 5283–5288.

[28] Y. Jiang, Y.P. Shang, H. Li, C. Zhang, J. Pan, Y.P. Bai, et al., Enhancing transglutaminase production of *Streptomyces mobaraensis* by iterative mutagenesis breeding with atmospheric and room-temperature plasma (ARTP), Bioresour. Bioprocess. 4 (2017) 37.

[29] X. Yin, Y. Li, J. Zhou, S. Rao, G. Du, J. Chen, et al., Enhanced production of transglutaminase in *Streptomyces mobaraensis* through random mutagenesis and site-directed genetic modification, J. Agric. Food Chem. 69 (2021) 3144–3153.

[30] S.S. Park, B.J. Ko, B.G. Kim, Mass spectrometric screening of transcriptional regulators using DNA affinity capture assay, Anal. Biochem. 344 (2005) 152–154.

[31] X.M. Mao, S. Luo, R.C. Zhou, F. Wang, P. Yu, N. Sun, et al., Transcriptional regulation of the daptomycin gene cluster in *Streptomyces roseosporus* by an autoregulator, AtrA. J. Biol. Chem. 290 (2015) 7992–8001.

[32] S. Qu, Q. Kang, H. Wu, L. Wang, L. Bai, Positive and negative regulation of GlnR in validamycin A biosynthesis by binding to different loci in promoter region, Appl. Microbiol. Biotechnol. 99 (2015) 4771–4783.

[33] A. Romero-Rodríguez, I. Robledo-Casados, S. Sánchez, An overview on transcriptional regulators in *Streptomyces*, Biochim. Biophys. Acta 1849 (2015) 1017–1039.

[34] Y. Ohnishi, S. Kameyama, H. Onaka, S. Horinouchi, The A-factor regulatory cascade leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor, Mol. Microbiol. 34 (1999) 102–111.

[35] M.T. López-García, I. Santamarta, P. Liras, Morphological differentiation and clavulanic acid formation are affected in a *Streptomyces clavuligerus adpA*-deleted mutant, Microbiol 156 (2010) 2354–2365.

[36] M. Komatsu, T. Uchiyama, S. Omura, D.E. Cane, H. Ikeda, Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 2646–2651.

[37] Y.L. Du, S.Z. Li, Z. Zhou, S.F. Chen, W.M. Fan, Y.Q. Li, The pleitropic regulator AdpAch is required for natamycin biosynthesis and morphological differentiation in *Streptomyces chattanoogensis*, Microbiol 157 (2011) 1300–1311.

[38] P. Yu, Q.T. Bu, Y.L. Tang, X.M. Mao, Y.Q. Li, Bidirectional regulation of AdpAch in controlling the expression of *scnRI* and *scnRII* in the natamycin biosynthesis of *Streptomyces chattanoogensis* L10, Front. Microbiol. 9 (2018) 1–11.

[39] T. Higashi, Y. Iwasaki, Y. Ohnishi, S. Horinouchi, A-factor and phosphate depletion signals are transmitted to the grixazone biosynthesis genes via the pathway-specific transcriptional activator GriR, J. Bacteriol. 189 (2007) 3515–3524.

[40] H. Yamazaki, Y. Ohnishi, S. Horinouchi, An A-factor-dependent extracytoplasmic function sigma factor (σ(AdsA)) that is essential for morphological development in *Streptomyces griseus*, J. Bacteriol. 182 (2000) 4596–4605.

[41] K.J. ya, S. A, H. Yamazaki, Y. Ohnishi, S. Horinouchi, Control by A-factor of a metalloendopeptidase gene involved in aerial mycelium formation in *Streptomyces griseus*, J. Bacteriol. 184 (2002) 6016–6025.

[42] H. Yamazaki, Y. Takano, Y. Ohnishi, S. Horinouchi, *amfR*, an essential gene for aerial mycelium formation, is a member of the AdpA regulon in the A-factor regulatory cascade in *streptomyces griseus*, Mol. Microbiol. 50 (2003) 1173–1187.

[43] H. Yamazaki, Y. Ohnishi, S. Horinouchi, Transcriptional switch on of *ssgA* by A-factor, which is essential for spore septum formation in *Streptomyces griseus*, J. Bacteriol. 185 (2003) 1273–1283.

[44] J.Y. Kato, W.J. Chi, Y. Ohnishi, S.K. Hong, S. Horinouchi, Transcriptional control by A-factor of two trypsin genes in *Streptomyces griseus*, J. Bacteriol. 187 (2005) 286–295.

[45] A. Tomono, Y. Tsai, Y. Ohnishi, S. Horinouchi, Three chymotrypsin genes are members of the AdpA regulon in the A-factor regulatory cascade in *Streptomyces griseus*, J. Bacteriol. 187 (2005) 6341–6353.

[46] H. Hara, Y. Ohnishi, S. Horinouchi, DNA microarray analysis of globalgene regulation by A-factor in *Streptomyces griseus*, Microbiol 155 (2009) 2197–2210.

[47] G. Akanuma, H. Hara, Y. Ohnishi, S. Horinouchi, Dynamic changes in the extracellular proteome caused by absence of a pleiotropic regulator AdpA in *Streptomyces griseus*, Mol. Microbiol. 73 (2009) 898–912.

[48] A. Higo, H. Hara, S. Horinouchi, Y. Ohnishi, Genome-wide distribution of AdpA, a global regulator for secondary metabolism and morphological differentiation in streptomyces, revealed the extent and complexity of the AdpA regulatory network, DNA Res. 19 (2012) 259–273.

[49] H. Yamazaki, A. Tomono, Y. Ohnishi, S. Horinouchi, DNA-binding specificity of AdpA, a transcriptional activator in the A-factor regulatory cascade in *Streptomyces griseus*, Mol. Microbiol. 53 (2004) 555–572.

[50] Y. Ohnishi, H. Yamazaki, J.Y. Kato, A. Tomono, S. Horinouchi, AdpA, a central transcriptional regulator in the A-factor regulatory cascade that leads to morphological development and secondary metabolism in *Streptomyces griseus*, Biosci. Biotechnol. Biochem. 69 (2005) 431–439.

[51] Y.D. Hindra, Q. Teng, D.L. Bin, I. Crnovčić, T. Huang, et al., Genome mining of *Streptomyces mobaraensis* DSM 40847 as a bleomycin producer providing a biotechnology platform to engineer designer bleomycin analogues, Org. Lett. 19 (2017) 1386–1389.

[52] L. Lorand, L.B. Weissmann, D.L. Epel, J. Bruner Lorand, Role of the intrinsic transglutaminase in the Ca^{2+} mediated crosslinking of erythrocyte proteins, Proc. Natl. Acad. Sci. U. S. A. 73 (1976) 4479–4481.

[53] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.

[54] G.Y. Tan, Y. Peng, C. Lu, L. Bai, J.J. Zhong, Engineering validamycin production by tandem deletion of γ-butyrolactone receptor genes in *Streptomyces hygroscopicus* 5008, Metab. Eng. 28 (2015) 74–81.

[55] T. Umeyama, P.C. Lee, S. Horinouchi, Protein serine/threonine kinases in signal transduction for secondary metabolism and morphogenesis in *Streptomyces*, Appl. Microbiol. Biotechnol. 59 (2002) 419–425.

[56] J.B. Stock, A.J. Ninfa, A.M. Stock, Protein phosphorylation and regulation of adaptive responses in bacteria, Microbiol. Rev. 53 (1989) 450–490.

[57] T.G. Patton, S.J. Yang, K.W. Bayles, The role of proton motive force in expression of the *Staphylococcus aureus cid* and *lrg* operons, Mol. Microbiol. 59 (2006) 1395–1404.

[58] X. Bu, J. Weng, B. He, M. Xu, J. Xu, A novel AdpA homologue negatively regulates morphological differentiation in *Streptomyces xiamenensis* 318, Appl. Environ. Microbiol. 85 (2019) e03107–e03118.

[59] R.H.N. Williams, D.E. Whitworth, The genetic organisation of prokaryotic two-component system signalling pathways, BMC Genom. Electron. Resour. 11 (2010) 720.

[60] J.K. Cheung, J.I. Rood, Erratum: the VirR response regulator from *Clostridium perfringens* binds independently to two imperfect direct repeats located upstream of the *pfoA* promoter, J. Bacteriol. 182 (2000) 57–66.

[61] D.J. Sidote, C.M. Barbieri, T. Wu, A.M. Stock, Structure of the *Staphylococcus aureus* AgrA LytTR domain bound to DNA reveals a beta fold with an unusual mode of binding, Structure 16 (2008) 727–735.

[62] A.N. Nikolskaya, M.Y. Galperin, A novel type of conserved DNA-binding domain in the transcriptional regulators of the AlgR/AgrA/LytR family, Nucleic. Acids. Res. 30 (2002) 2453–2459.