Bacillus licheniformis global nitrogen homeostatic regulator TnrA is a direct repressor of *pgsBCAA* transcription in Poly-γ-glutamic acid synthesis

Running Title: TnrA represses *pgsB* transcription in *B. licheniformis*

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Abstract Poly-γ-glutamic acid (γ-PGA) is a multifunctional and naturally occurring biopolymer made from D- and L-glutamate as monomers, which is mainly produced by *Bacillus*. Few reports have been focused on the regulation network of γ-PGA synthesis in recent years. In this study, we have demonstrated that *Bacillus licheniformis* global nitrogen homeostatic regulator TnrA is a direct repressor of γ-PGA synthase PgsBCAA in γ-PGA synthesis. First, our results confirmed that TnrA repressed γ-PGA synthesis, deficiency of *tnrA* led to a 22.03% increase of γ-PGA production, and the γ-PGA yield was decreased by 19.02% in the TnrA overexpression strain. Transcriptional level assay illustrated that the γ-PGA synthase gene cluster *pgsBCAA* transcriptional level were increased in the *tnrA* deficient strain WXΔtnrA, indicating that γ-PGA synthase PgsBCAA was negatively regulated by TnrA. Furthermore, electrophoretic mobility shift assay (EMSA) and enzyme expression assays confirmed that TnrA directly repressed *pgsBCAA* expression by binding to *pgsBCAA* promoter, and the TnrA-binding site "CGTCGTCTTCTGTTACA" in the *pgsBCAA* promoter was identified by sequence and software analysis. Finally, computer analysis confirmed that the transcription regulations of γ-PGA synthase PgsBCAA by TnrA were highly conserved in other well-studied *Bacillus* species (*B. licheniformis*, *Bacillus subtilis* and *Bacillus amyloliquefaciens*). Collectively, our results implied that TnrA was a direct repressor for *pgsBCAA* expression in γ-PGA synthesis, and this research provided a novel regulatory mechanism underlying γ-PGA synthesis, and a new approach that deficiency of *tnrA* increases γ-PGA production.

Importance

γ-PGA is an important biopolymer with many applications, which is mainly produced by *Bacillus* species. Glutamic acid is the precursor for γ-PGA synthesis, which is catalyzed by the γ-PGA synthase PgsBCAA. Previously, the expression of PgsBCAA was reported to be regulated by ComA-ComP and DegS-DegU, DegQ and SwrA systems, however, few researches were focused on the regulation network of γ-PGA synthesis in recent years. In our research, the γ-PGA synthase PgsBCAA was confirmed to be negatively regulated by the nitrogen metabolism regulator TnrA, and the TnrA binding site in the *pgsBCAA* promoter was identified in *B. licheniformis* WX-02. Furthermore, computer analysis implied that TnrA-mediated regulation effect on *pgsBCAA* expression was highly conserved in *Bacillus*. Collectively, our research provided a novel regulatory mechanism underlying γ-PGA synthesis, and a new approach that deficiency of *tnrA* increases γ-PGA production.

Keywords: *Bacillus licheniformis*; Poly-γ-glutamic acid; nitrogen regulator TnrA; transcription regulatory; γ-PGA synthase PgsBCAA

Introduction

Poly-γ-glutamic acid (γ-PGA) is a multifunctional and naturally occurring biopolymer made from D- and L-glutamate as monomers (1). Due to its owns the features of cation chelating, hygroscopicity, water-solubility, non-toxicity and biodegradability, γ-PGA has been used as various materials such as drug carriers, food preservatives, metal ion chelating agents, highly water absorbable hydrogels and fertilizer accelerators, and applied in the areas of medicine, food, water treatment, cosmetics and agriculture etc (2, 3).

Generally, *Bacillus* species were regarded as the efficient γ-PGA producers, and the precursor, *in vivo* glutamic acid, was applied for γ-PGA biosynthesis under the catalysis of γ-PGA synthase complex PgsBCAA (2). Among this gene cluster, gene *pgsB* acts as the main function role of γ-PGA synthase, whereas *pgsC* owns the function of γ-PGA secretion, and *pgsAA* is necessary for the next monomer addition and transportation of γ-PGA through the cell membrane (4). Previously, the γ-PGA synthesis was reported to be regulated by the two-component systems ComA-ComP and DegS-DegU, DegQ and SwrA systems (5). Among them, ComA-ComP and DegS-DegU, DegQ were involved in the quorum sensing, osmolarity, phase variation signals, while SwrA appears to act at the post-transcriptional level. Based on the previous researches, the regulation of γ-PGA synthesis by DegQ was well investigated, and the γ-PGA synthesis capability was abolished in the *degQ* mutant strain. Phosphorylated DegU (DegU-P) and SwrA up-regulated the expression level of *pgs* operon for γ-PGA synthesis (6, 7), and no more in-depth study was conducted

to resolve this mechanism. Recent years, several researches were focused on the improvement of γ-PGA production by metabolic engineering strategies (8-10), however, few works were conducted to elaborate the regulation network of γ-PGA synthesis, thereby, it was unclear that whether the γ-PGA synthase PgsBCAA was regulated by other regulators.

The global nitrogen homeostatic regulator TnrA is known as a member of MerR family transcriptional factor, and it could both activate and repress the expression of many genes under nitrogen limited condition (11). Generally, TnrA up-regulated the expression levels of genes encoding ammonium transport (*amtB-glnK*) (12), nitrate assimilation (*nasAB*) (13), nitrite assimilatory enzymes (*nasDEF*) etc (14), and also exerted negative effects on the expression of *glnRA* (encoding for glutamine synthase) (15), *gltAB* (encoding for glutamic acid synthase) (16), *ilv-leu* (encoding for branched-chain amino acids synthase) (17), *degU* (encoding for two-component system DegS-DegU) etc (18). Previously, the chromatin immunoprecipitation coupled with hybridization to DNA tiling arrays (ChIP-on-chip) was applied to identify the TnrA binding sites at the genome scale of *B. subtilis*, and their results provided that TnrA binds reproducibly to 42 regions. Among them, 35 TnrA primary regulons were confirmed by real time in vivo transcriptional profiling using firefly luciferase. In their research, a predicted TnrA box has been implied in the promoter region of γ-PGA synthase gene *pgsB* (19), however, no in-depth research was conducted to analyze the relationship between TnrA and *pgsBCAA* expression.

Bacillus licheniformis WX-02 was proven as an efficient γ-PGA producer (20),

and our previous research found that the genes related to glutamic acid synthesis were activated by 6% NaCl addition (21), and the glutamic acid synthesis capabilities were also improved by the physicochemical stresses such as heat, osmotic and alkaline etc (22-24). Furthermore, several metabolic engineering strategies have been conducted to improve the γ-PGA production (8, 25, 26). In this study, we demonstrated that *Bacillus licheniformis* global nitrogen homeostatic regulator TnrA is a direct repressor of *pgsBCAA* transcription in γ-PGA synthesis, and the TnrA-mediated regulation effect on *pgsBCAA* expression was highly conserved in *Bacillus*.

Results

Deficiency of *tnrA* increased γ-PGA production

TnrA is known as a global nitrogen regulator in *B. subtilis*. In order to test the function role of TnrA on γ-PGA production in *B. licheniformis*, the *tnrA* deficient and overexpression strains were constructed based on *B. licheniformis* WX-02, and the recombinant strains were named as WXΔtnrA andWX/pHY-tnrA, respectively. Then, these recombinants, as well as the control strains WX-02 and WX/pHY300, were cultivated in the γ-PGA production medium, and the γ-PGA yields at the end of fermentation (32 h) were measured to evaluate the effects of *tnrA* deficiency and overexpression on the γ-PGA production. Based on our results of **Fig. 1**, WXΔtnrA produced 38.37 g/L γ-PGA, increased by 22.03% compared with that of WX-02 (31.45 g/L), indicating that deficiency of *tnrA* improved γ-PGA production. Meanwhile, overexpression of TnrA led to a 19.02% decrease of γ-PGA yield,

124 compared to WX/pHY300.

125 Furthermore, the γ-PGA yields, cell biomass, residue concentrations of glucose
126 and glutamic acid of these strains were measured during γ-PGA production. Our
127 results implied that the γ-PGA yields of WXΔtnrA were higher than those of WX-02
128 throughout the whole fermentation process, and the maximum γ-PGA yield was
129 increased by 22.03%. Glucose and glutamic acid consumption rates of WXΔtnrA
130 were 9.13% and 11.34% higher than those of WX-02, respectively. Additionally,
131 since the deficiency of *tnrA* affects the expression levels of nitrogen utilization genes
132 (19, 27), which led to the low cell growth of WXΔtnrA during the γ-PGA
133 fermentation. The specific γ-PGA yield of WXΔtnrA was 6.11g/g $_{DCW}$, increased by
134 42% compared with that of WX-02 (4.31g/g $_{DCW}$). Meanwhile, overexpression of *tnrA*
135 decreased the γ-PGA production, as well as the glucose and glutamic acid
136 consumption rates, and the cell biomass increased obviously in the *tnrA*
137 overexpression strain (**Fig. 2**). Collectively, our results demonstrated that the nitrogen
138 regulator TnrA repressed the synthesis of γ-PGA, and deficiency of *tnrA* increased
139 γ-PGA yield.

140

141 **The transcriptional levels of γ-PGA synthase genes *pgsBCAA* were increased in**
142 **the *tnrA* deficient strain**

143 Then, the transcriptional level of genes involved in glucose metabolism and
144 γ-PGA biosynthesis were determined during γ-PGA production. As shown in **Fig. 3**,
145 no *tnrA* transcriptional level was determined in WXΔtnrA, indicating that *tnrA* was

deleted successfully. The transcriptional levels of γ-PGA synthesis genes *pgsB*, *pgsC* and *pgsAA* were all increased in WX△tnrA (5.53-fold, 4.64-fold and 4.08-fold, respectively), and the transcription levels of their regulator genes, *degU*, was increased by 1.68-fold in the *tnrA* deficient strain. The glutamate synthase gene *gltA* was increased by 2.56-fold in WXΔtnrA. Moreover, the transcriptional levels of glucose-6-phosphate isomerase gene *pgi* and glyceraldehyde-3-phosphate dehydrogenase gene *gapA* in the glycolytic pathway were increased obviously, as well as the citrate synthase gene *citB* and isocitrate dehydrogenase gene *icd* in tricarboxylic acid (TCA) cycle. The glucose 6-phosphate dehydrogenase gene *zwf* in pentose phosphate pathway showed no significant changes in *tnrA* deficient strain. Furthermore, the genes, *alsS* and *alsD*, which responsible for the synthesis of main byproducts acetoin and 2,3-butandieol, were all decreased in WXΔtnrA, indicating that the deficiency of *tnrA* repressed the overflow metabolism, which were beneficial for γ-PGA synthesis.

Identification of TnrA binding sites in the *pgsBCAA* promoter

Previous research has implied that there might be a predicted TnrA box in the *pgsB* promoter of *B. subtilis* (19). To verify whether TnrA regulated the *pgsBCAA* promoter directly or not in *B. licheniformis* WX-02, electrophoretic mobility shift assays (EMSAs) were performed. We expressed His_6-tagged TnrA in *E. coli* BL21(DE3) and purified, and the SDS-PAGE results in **Fig. 4A** demonstrated that the TnrA protein was induced and purified successfully. Then, the DNA probes of

168 *pgsBCAA* promoter (form -300 to +50) were amplified, and the purified DNA probes
169 were incubated with the purified His_6-tagged TnrA according to the instruction
170 manual of EMSA kit. As shown in **Fig. 4B**, a TnrA-P_{pgsB} complex was formed in a
171 concentration-dependent manner, indicating that TnrA could bind to the promoter of
172 *pgsBCAA*.

173 Previously, the TnrA box was proven to be a 17-bp interrupted, inverted repeat
174 sequence "TGTNANAWWWTNTNACA" in *B. subtilis* (28). In this research, based
175 on the conserved sequence of TnrA box and MEME software screening, the suspected
176 TnrA binding site in *pgsBCAA* promoter, "CGTCGTCTTCTGTTACA", was screened,
177 which is outside of the "-10" and "-35" regions of *pgsBCAA* promoter (**Fig. 4C**). Then,
178 the sequence of predicted TnrA box was further deleted in the *pgsBCAA* promoter,
179 and formed the promoter $P_{pgsB'}$. Our results confirmed that there was no TnrA-$P_{pgsB'}$
180 complex formed in the EMSA assay, even though 200 ng TnrA was applied **(Fig. 4B)**.
181 Thus, it was confirmed that TnrA directly band the *pgsBCAA* promoter, and the TnrA
182 box "CGTCGTCTTCTGTTACA" in *pgsBCAA* promoter has been identified in *B.*
183 *licheniformis* WX-02.

184

185 **_pgsBCAA_ promoter is negatively regulated by TnrA**

186 Furthermore, to investigate whether *pgsBCAA* was negatively or positively
187 regulated by TnrA *in vivo*, the nattokinase expression assay was performed. Firstly, to
188 exclude the interference of regulator DegU, which has been confirmed as the direct
189 positive activator of *pgsBCAA* in the previous research (6, 7), we deleted gene *degU*

in WX-02 and WXΔtnrA, and resulting in the mutant strains WXΔdegU and WXΔtnrAΔdegU. Then, the nattokinase expression vector mediated by P_{pgsB} were constructed, and electro-transferred into WXΔdegU and WXΔtnrAΔdegU, respectively. Based on our results of **Fig. 5**, the nattokinase activities of WXΔtnrAΔdegU were higher those of WXΔdegU under the condition of P_{pgsB} promoter, and the maxmium activity of WXΔtnrAΔdegU was 26.39 FU/mL, increased by 45.19% compared with that of WXΔdegU (18.18 FU/mL). Also, the specific nattokinase activity of WXΔtnrAΔdegU/pPgsBSacCNK was 2.57 FU/OD_{600}, increased by 91.66% compared to WXΔdegU/pPgsBSacCNK (1.34 FU/OD_{600}) (**Fig. 5A**). Thus, these results indicated that deficiency of *tnrA* might improve the expression of *pgsBCAA* promoter, which led to the improvement of nattokinase activity in the WXΔtnrAΔdegU/pPgsBSacCNK. Additionally, to exclude the interference of *tnrA* deficiency on the cell growth, which might affect nattokinase production, the nattokinase vector mediated by P43 prmoter was applied, and electro-transferred into WXΔdegU and WXΔtnrAΔdegU, respectively. Our results implied that the nattokinase activities produced by WXΔtnrAΔdegU/pP43SacCNK were lower than those of WXΔdegU/pP43SacCNK throughout the fermentation process (**Fig. 5B**), and this might due to that deletion of *tnrA* led to the reduction of cell growth, which further influence nattokinase production (27). Collectively, these above results illustrated that TnrA negatively regulated the transcription of *pgsBCAA*, and the nattokinase activity mediated by P_{pgsB} was improved in the *tnrA* deficient strain.

The transcriptional regulation of *pgsBCAA* by TnrA is highly conserved in *Bacillus*

Finally, we investigated whether TnrA directly regulate the transcription of γ-PGA synthase PgsBCAA or not in other *Bacillus* species. We screened the γ-PGA synthase gene cluster *pgsBCAA* in three well-studied *Bacillus* species (*B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*) according to the genome annotation from NCBI database, and searched the TnrA binding motif in their promoters regions. Sequence and software analyses indicated that TnrA binding sites were located in the *pgsBCAA* promoters of various *Bacillus* species: 14/15 *B. licheniformis*, 75/76 *B. subtilis*, 21/22 *B. amyloliquefaciens*. Meanwhile, the TnrA boxes were predicted in the corresponding promoters, which all located outside of the "-10" and "-35" regions of *pgsBCAA* promoters (**Table 3**). Thus, these results suggested that γ-PGA synthase PgsBCAA regulated by nitrogen regulator TnrA was highly conserved in *Bacillus*.

Discussion

γ-PGA is an important biopolymer with many applications (29). Recently, several researches have been focused on the strategies of metabolic engineering to improve γ-PGA production, including eliminating the byproduct synthesis pathways, strengthening the γ-PGA pathways, manipulating ATP supply and NADPH generation (8, 10, 25, 30). However, the regulation network of γ-PGA synthesis has not been well analyzed, besides the regulators ComA~ComP, DegS~U, DegQ and SwrA (**Fig. 6**). In this study, it was confirmed that the nitrogen regulator TnrA was a

234 directly repressor of γ-PGA synthase PgsBCAA, and deficiency of *tnrA* led to a 22.03%
235 increase of γ-PGA yield.

236 γ-PGA was mainly produced by *Bacillus* species. *In vivo* glutamic acid is served
237 as the precursor for γ-PGA synthesis, and nitrate addition could strengthen γ-PGA
238 synthesis by our previous research, and ammonium salt was also acted as the
239 important resource for γ-PGA synthesis (24, 31). Thus, nitrogen metabolism played
240 an important role in γ-PGA synthesis. Since TnrA is a global nitrogen metabolism
241 regulator in *Bacillus*, which acted both as the activator or repressor for nitrogen
242 metabolism genes (11), thus, the regulator TnrA might also affect γ-PGA synthesis.
243 Based on the previous research, a suspected TnrA box was predicted in the *pgsB*
244 promoter in *B. subtilis* by Chip-on-chip (19), and our research confirmed that the
245 γ-PGA synthase complex PgsBCAA were directly regulated by the nitrogen regulator
246 TnrA, and the related genes (*pgsB*, *pgsC* and *pgsAA*) transcriptional levels were all
247 increased obviously in the *tnrA* deficient strain, which led to the improvement of
248 γ-PGA yield. In addition, TnrA was also proven as the repressor of the genes *gltAB*
249 and *degU* (16), which were responsible for glutamate synthase and regulation of
250 γ-PGA synthase in *Bacillus*. Based on our results, *gltA* and *degU* transcriptional level
251 were increased obviously in the *tnrA* deficient strain, and this might be another reason
252 that the γ-PGA yield produced by WXΔtnrA was higher than that of WX-02, and the
253 proposed regulatory network of TnrA in γ-PGA biosynthesis in *B. licheniformis*
254 WX-02 was provided in **Fig. 6**. Additionally, the nitrogen regulators GlnR and CodY
255 acted as the similar features compared to TnrA, however, whether these regulators

affect γ-PGA synthesis or not is still unknown recent now.

Previously, the γ-PGA synthesis was reported to be regulated by the two-component systems ComA-ComP and DegS-DegU, DegQ and SwrA systems (5). The γ-PGA synthesis capability was abolished in the *degQ* mutant strain, and phosphorylated DegU (DegU-P) and SwrA could up-regulate the expression level of *pgs* operon for γ-PGA synthesis (6, 7). Since TnrA was reported to be the repressor for *degU* expression (18), the transcriptional level of *degU* was increased by 1.68-fold in the *tnrA* deficient strain, thus, TnrA could affect γ-PGA synthesis through up-regulating the expression levels of *degU*, and the increase of *degU* transcriptional level was beneficial for γ-PGA synthesis. In our work, in order to exclude the interference of regulator DegU, we deleted gene *degU* in WX-02 and WXΔtnrA, and further analyze the regulation mechanism of *pgsBCAA* promote by TnrA in the *degU* mutant strains. Our results confirmed that nattokinase activity produced by WXΔtnrAΔdegU/pPgsBSacCNK was increased obviously compared to WXΔdegU/pPgsBSacCNK. Additionally, the TnrA-binding site "CGTCGTCTTCTGTTACA" was identified in the *pgsBCAA* promoter of *B. licheniformis* WX-02. Therefore, our results confirmed that TnrA could directly repress the transcription of *pgsBCAA* by binding to its promoter, in addition to the indirect effect of DegU. Nonetheless, the in-depth research is worth to explore that which pathway plays the major role for the improvement of γ-PGA yield in the *tnrA* mutant strain.

Bacillus species were the important industrial production strains (32), and they

could produce various kinds of bio-chemical products (33), also, *Bacillus* could be acted as the host strains for protein expression (34). Among them, *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* are the three well-studied *Bacillus* species, and they are also the main producers for γ-PGA synthesis (29). Furthermore, these species have a high degree of homology, *B. licheniformis* owns a nearly 75% identity with *B. subtilis* at the genome-level, and *B. amyloliquefaciens* has been regarded as a subspecies of *B. subtilis*, which genome identity is more than 80% with *B. subtilis*. Thus, these species might belong to the same branch in evolutionary terms. Based on our results, the TnrA boxes of *pgsBCAA* promoters were predicted in the most of these strains (**Table 3**), thus, our results suggested that the regulation of γ-PGA synthase PgsBCAA by TnrA might be conserved in *Bacillus*.

In conclusion, the regulation model of γ-PGA synthase PgsBCAA by nitrogen regulator TnrA was analyzed in this study. Based on our results, the γ-PGA synthesis was negatively regulated by TnrA, and deficiency of *tnrA* led to a 22.03% increase of γ-PGA yield. Transcriptional analysis confirmed that *pgsBCAA* expression level was all increased in the *tnrA* deficient strain, and EMSA and enzyme expression assays confirmed that the expression of *pgsBCAA* was directly negatively regulated by the regulator TnrA, and the TnrA-binding site was identified in the *pgsBCAA* promoter of *B. licheniformis*. Finally, we demonstrated that the TnrA-binding sites in the *pgsBCAA* promoters were highly conserved in *Bacillus*. Taken together, our results implied that TnrA was a direct repressor for *pgsBCAA* expression in γ-PGA synthesis, and our research provided a novel regulatory mechanism underlying γ-PGA synthesis,

and a new approach that deficiency of *tnrA* increases γ-PGA production.

Materials and methods

Bacterial strains and plasmids

The strains and plasmids used in this research were provided in **Table 1**. *B. licheniformis* WX-02 acts as the original strain for constructing mutants. The plasmid T_2(2)-Ori was applied to construct the *tnrA* knockout vector T_2-tnrA. The TnrA overexpression vector pHY-tnrA was obtained based on pHY300. All premiers used for strain construction were provided in **Table 2**.

Medium and cultivate condition

LB medium (10 g/L Tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2) was served as the basic medium for strain cultivation, and responsible antibiotic (20 μg/mL kanamycin, 50 μg/mL ampicillin or 20 μg/mL tetracycline) was added into the medium when necessary. The seed culture of *B. licheniformis* was prepared in a 250 mL flask with 50 mL LB medium, and incubated at the rotatory shaker with 180 rpm at 37ºC for 10-12 h until OD_{600} reached 4.0~4.5, and then transferred into the γ-PGA and nattokinase production medium, respectively. The γ-PGA production medium consisted of (per liter) 80 g glucose, 30 g glutamic acid, 10 g sodium citrate, 8 g NH_4Cl, 1 g $CaCl_2$, 1 g $K_2HPO_4{\cdot}3H_2O$, 1 g $MgSO_4{\cdot}7H_2O$, 1 g $ZnSO_4{\cdot}7H_2O$ and 0.15 g $MnSO_4{\cdot}7H_2O$ at pH 7.2(25). The nattokinase fermentation medium contained (per liter) 20 g glucose, 10 g peptone, 5 g yeast extract, 10 g NaCl, 10 g soy peptone, 10 g corn steep liquor, and 6 g $(NH_4)_2SO_4$, pH 7.2 (35). The fermentation conditions were the same as those of the seed culture.

322 **Construction of *tnrA* deletion strain**

323 The *tnrA* mutant strain was constructed according to our previous method (35).
324 Briefly, the up-stream and down-stream homologous arms of *tnrA* were amplified
325 with the corresponding premiers, and fused by Splicing-Overlapping-Extension PCR
326 (SOE-PCR). The fused fragments was then inserted into the plasmid T_2(2)-Ori at the
327 restriction sites *Xba*I and *Sac*I, diagnostic PCR and DNA sequence were used to
328 confirm that the recombinant plasmid (T_2-tnrA) was constructed successfully.

329 The vector T_2-tnrA was electro-transferred into *B. licheniformis* by the previous
330 reported method, verified by diagnostic PCR and plasmid extraction (36). The
331 positive transformants were cultivated in the LB medium with 20 μg/mL kanamycin
332 at 45ºC to promote the single-cross transformation for three generations, and then
333 transferred into the kanamycin-free medium at 37ºC for six generations. Then, the
334 kanamycin-sensitive colonies were verified by diagnostic PCR, and the mutant strain
335 was confirmed by DNA sequencing, named as WXΔtnrA.

336 **Construction of expression vectors**

337 The expression vectors were constructed via according to the following steps,
338 and the TnrA expression vector was served as an example. Briefly, the P43 promoter
339 of *B. subtilis* 168 (K02174.1), gene *tnrA* (3101408) and *amyL* terminator (FJ556804.1)
340 of *B. licheniformis* WX-02 were amplified using the corresponding primers, and fused
341 by SOE-PCR. The fused fragments were inserted into the expression vector
342 pHY300PLK at the restriction sites *Xba*I and *Eco*RI. Diagnostic PCR and DNA
343 sequence were used to confirm that the recombinant plasmid (pHY-tnrA) was

344 constructed successfully. Similarly, the nattokinase expression vector mediated by
345 *pgsB* promoter was constructed by the same method, named as pPgsBSacCNK, and
346 the nattokinase expression vector mediated by P43 promoter pP43SacCNK was
347 obtained in our previous research (37).

348 **Analytical methods**

349 Cell biomass was measured based on cell dry weight. The γ-PGA concentration
350 was determined by High performance liquid chromatography (HPLC) according to
351 the method described in our previous research (38). Glucose and glutamic acid
352 concentrations were measured by a SBA-40C bioanalyzer according to the manual
353 instruction (Academy of sciences, Shandong, China).

354 **Expression and purification of TnrA in *Escherichia coli***

355 The TnrA induced expression vector was constructed based on the following
356 steps. Briefly, the fragment of *tnrA* (3101408) was amplified from *B. licheniformis*
357 WX-02 using the corresponding premiers, and inserted into the induced vector
358 pET-28a at the restriction sites *Eco*RI and *Xho*I. The recombinant plasmid pET-TnrA
359 was verified by diagnostic PCR and DNA sequence. Expression and purification of
360 TnrA were performed as described previously (39). The purified proteins were
361 verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE),
362 and protein concentration was measured by a microplate reader (Bio-Rad, USA).

363 **Electrophoretic mobility shift assays**

364 The sequence of *pgsBCAA* promoter (Gene ID: 3028267, from -300 to +50) for
365 electrophoretic mobility shift assays (EMSAs) were amplified with gene-specific

primers containing 5'-biotin-modified universal primer. The PCR products were analyzed by agarose gel electrophoresis and purified using a PCR purification kit (Omega, USA). The concentration of biotin-labeled DNA probe was determined using a trace Spectrophotometer NanoDrop 2000 (Thermo, USA). For amplification the *pgsBCAA* promoter without the TnrA binding site ($P_{pgsB'}$), SOE-PCR was applied to fuse the upstream and downstream fragments of TnrA binding site, and formed the fragment $P_{pgsB'}$.

The EMSAs were carried out according to the manufacturer's protocol of the chemiluminescent EMSA kit (Beyotime Biotechnology, China). The binding reaction mixture containing 10 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 0.01% Nonidet P-40, 50 μg/mL poly(deoxyinosinic-deoxycytidylic) acid (poly(dI-dC)), and 10% glycerol. Biotin-labeled DNA probes were incubated individually with various concentrations of TnrA proteins at 25°C for 20 min. After binding, the samples were separated on a 4% nondenaturing PAGE gel in an ice-bath of 0.5×Tris-borate-EDTA (TBE) at 100 V, and trans-blotted to nylon membrane with mini trans-blot electrophoresis apparatus (Liuyi, China). Then, the membrane was treated by Chemiluminescent EMSA Kit (Beyotime, China) and analyzed with the MF-ChemiBIS (DNR Bio-imaging systems, Israel) (40).

Transcriptional level assay

The gene transcriptional levels of mutant strains were analyzed based on the previously reported method (8). In brief, total RNA was extracted by TRIzol® Reagent, and the trace DNA was digested by DNase I. The first stand of cDNA was amplified

388 by the Revert Aid First Strand cDNA Synthesis Kit (Thermo, USA). The 16S rRNA of
389 *B. licheniformis* WX-02 was used as the reference gene. The gene transcriptional
390 levels of recombinant strains were compared with those of the control strain after
391 being normalized to the reference gene 16S rRNA. All the experiments were
392 performed in triplicates.

393 **Nattokinase activity assay**

394 The nattokinase activity was measured by fibrin degradation method (35). In
395 brief, 0.4 mL fibrinogen solution (0.72%, w/v) and 1.4 mL Tris-HCl (50 mM, pH 7.8)
396 were mixed and incubated at 37ºC for 5 min, added 0.1 mL thrombin (20 U/mL) and
397 incubated at 37ºC for 10 min. The prepared fibrin-substrate solution was then mixed
398 with 0.1 mL nattokinase-containing broth, and incubated at 37ºC for 60 min and
399 shook every 20 min during the incubation. Then, 2 mL trichloroacetic acid (TCA)
400 solution (0.2 M) was added to stop the reaction. As a control, 0.1 mL
401 nattokinase-containing broth and 2 mL TCA solution (0.2 M) were added into the
402 prepared fibrin-substrate solution after incubating at 37ºC for 60 min. The samples
403 were centrifuged at 13,000 *g* for 10 min and measured the absorbance at 275 nm. One
404 unit nattokinase activity (FU) was defined as the amount of enzyme leading to 0.01
405 increase of absorbance in 1 min.

406 **Computational analysis**

407 The TnrA binding sites in the *pgsBCAA* promoters of various *Bacillus* strains
408 were identified by MEME/MAST tools (http://meme-suite.org/), according to the
409 conserved TnrA box reported in the previous research (40).

Statistical analyses

All samples were analyzed in triplicate, and the data were presented as the mean ± the standard deviation for each sample point. All data were conducted to analyze the variance at $P < 0.05$ and $P < 0.01$, and a *t* test was applied to compare the mean values using the software package Statistica 6.0 (35).

Competing interests

The authors declare that they have no competing interests.

Athour's contribution

D Cai and S Chen designed the study. D Cai and Y Chen carried out the molecular biology studies and construction of engineering strains. D Cai, Y Chen, S Wang and F Mo carried out the fermentation studies. D Cai, X Ma and S Chen analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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Supporting Information

All the primers sequences for RT-qPCR were listed in **Table S1**. These information were available free of charge via the Internet: http://aem.asm.org/.

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550 **Figure captions**

551 **Fig. 1: Effects of *tnrA* deficiency and overexpression on γ-PGA production.** Data
552 are represented as the means of three replicates and bars represent the standard
553 deviations, *, $P < 0.05$; and **, $P < 0.01$ indicate the significance levels between
554 recombinant strains and control strain.

555

556 **Fig. 2: Fermentation process curve of *B. licheniformis* WX-02, WXΔtnrA,**
557 **WX/pHY300 and WX/pHY-TnrA. A**: γ-PGA; **B**: Cell biomass; **C**: Glucose
558 concentrations; **D**: Glutamic acid concentrations.

559

560 **Fig. 3: Transcriptional level analysis. A:** Effects of *tnrA* deficiency on the relative
561 transcriptional levels of genes in γ-PGA biosynthesis; **B:** Effects of *tnrA* deficiency on
562 the relative transcriptional levels of genes in glucose metabolism.

563

564 **Fig. 4: EMSA assay and identification of the TnrA binding site in the *pgsBCAA***
565 **promoter. A: Expression and purification of TnrA in BL21(DE3);** Lane 1: The
566 protein Marker (170 DKa, 130 DKa, 100 DKa, 70 DKa, 55 DKa, 40 DKa, 35 DKa, 25
567 DKa, 15 DKa,), Lane 2: The total intracellular protein of BL21/pET-TnrA before
568 induction, Lane 3: The total intracellular protein of BL21/pET-TnrA induced by IPTG,
569 Lane 4: The purified TnrA protein. The arrow indicates the purified TnrA (13.17
570 KDa); **B: EMSA assay of the purified TnrA protein and *pgsBCAA* promoter.**
571 Lane 1: EMSA of TnrA protein (200 ng) with biotin-labeled *pgsBCAA* promoter $P_{pgsB'}$,

572 which lacking out of the TnrA binding site "CGTCGTCTTCTGTTACA", as the
573 negative group; Lane 2-4: EMSA of different TnrA concentrations (0, 100 ng, 200 ng)
574 with biotin-labeled *pgsBCAA* promoter (-300 to +50 bp upstream and downstream of
575 the translational start), and the arrow indicates the mixture of TnrA with *pgsB*
576 promoter. **C: Identification of the TnrA-binding site in the *pgsBCAA* promoter of**
577 ***B. licheniformis* WX-02.**

578

579 **Fig. 5: Confirmation of the regulation model of γ-PGA biosynthesis by TnrA via**
580 **nattokinase expression assay. A:** nattokinase activity produced by
581 WXΔdegU/pPgsBSacCNK and WXΔtnrAΔdegU/pPgsBSacCNK; **B:** nattokinase
582 activity produced by WXΔdegU/pP43SacCNK and WXΔtnrAΔdegU/pP43SacCNK.

583

584 **Fig. 6 The proposed regulatory network of TnrA in γ-PGA biosynthesis in *B.***
585 ***licheniformis*** (The black lines indicate the previously proven regulatory mechanisms
586 in *Bacillus*, the cyan line indicates the regulatory mechanism confirmed in this
587 research)**.**

588

Table 1 The strains and plasmids used in this research

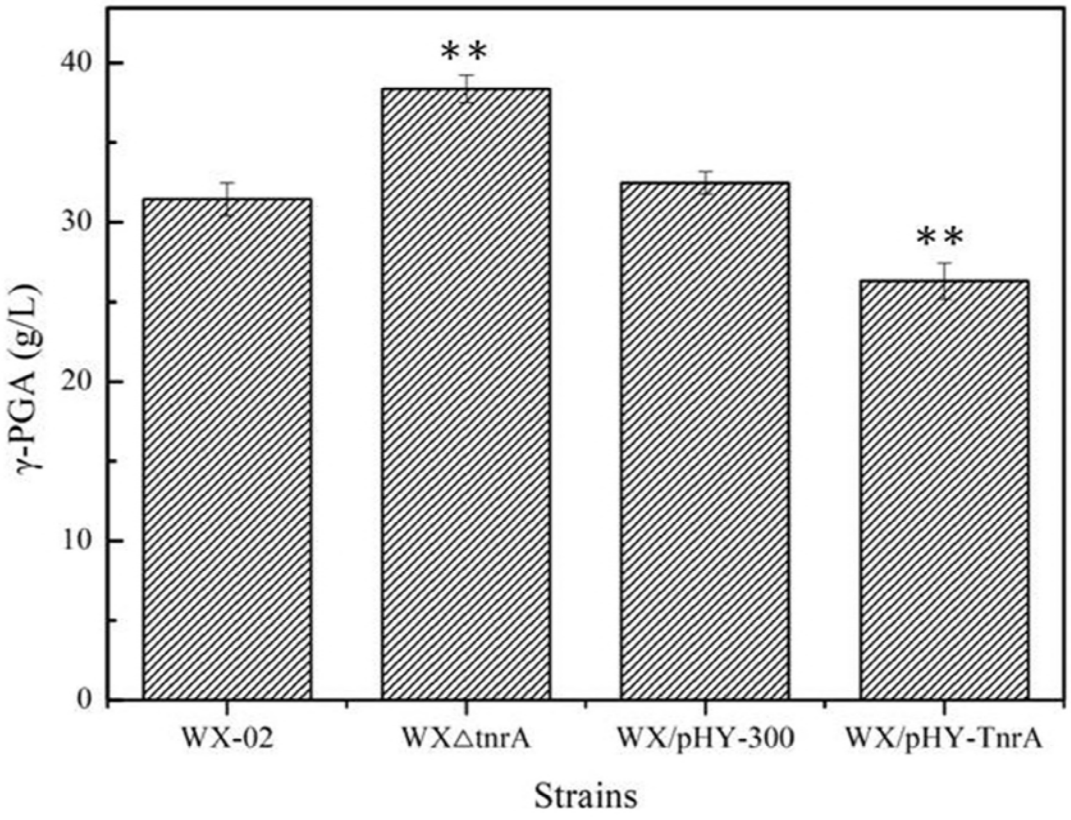
Strains or plasmids	Description	Source of reference
Bacillus licheniformis		
WX-02	Poly γ-glutamate productive strain (CCTCC M208065)	CCTCC
WXΔtnrA	WX-02 (ΔtnrA)	This study
WX/pHY300	WX-02 harboring pHY-300, as control	This study
WX/pHY-TnrA	WX-02 harboring pHY-TnrA	This study
WXΔdegU	WX-02 (ΔdegU)	This study
WXΔtnrAΔdegU	WX-02 (ΔtnrA; ΔdegU)	This study
WXΔdegU/pP43SacCNK	WXΔdegU harboring pP43SacCNK	This study
WXΔdegU/pPgsBSacCNK	WXΔdegU harboring pPgsBSacCNK	This study
WXΔtnrAΔdegU/pP43SacCNK	WXΔtnrAΔdegU harboring pP43SacCNK	This study
WXΔtnrAΔdegU/pPgsBSacCNK	WXΔtnrAΔdegU harboring pPgsBSacCNK	This study
Plasmids		
T_2(2)-Ori	*Bacillus* knockout vector; Kan^r	This study
T_2-tnrA	T2(ori)-tnrA(A+B); to knock out *tnrA*	This study
T_2-degU	T2(ori)-degU(A+B); to knock out *degU*	This study
pHY300PLK	*E. coli-B. licheniformis* shuttle vector, Ap^r (*E. coli*), Tc^r (*E. coli* and *B. licheniformis*)	This study
pHY-tnrA	pHY300PLK containing P43 promoter, gene *tnrA* and *amyL* terminator	This study
pPgsBSacCNK	pHY300PLK containing *pgsB* promoter, signal peptide of SacB, gene *aprN* and *amyL* terminator	This study
pP43SacCNK	pHY300PLK containing P43 promoter, signal peptide of SacB, gene *aprN* and *amyL* terminator	This study
pET-28a	Induced expression vector	This study
pET-TnrA	TnrA induced expression vector	This study

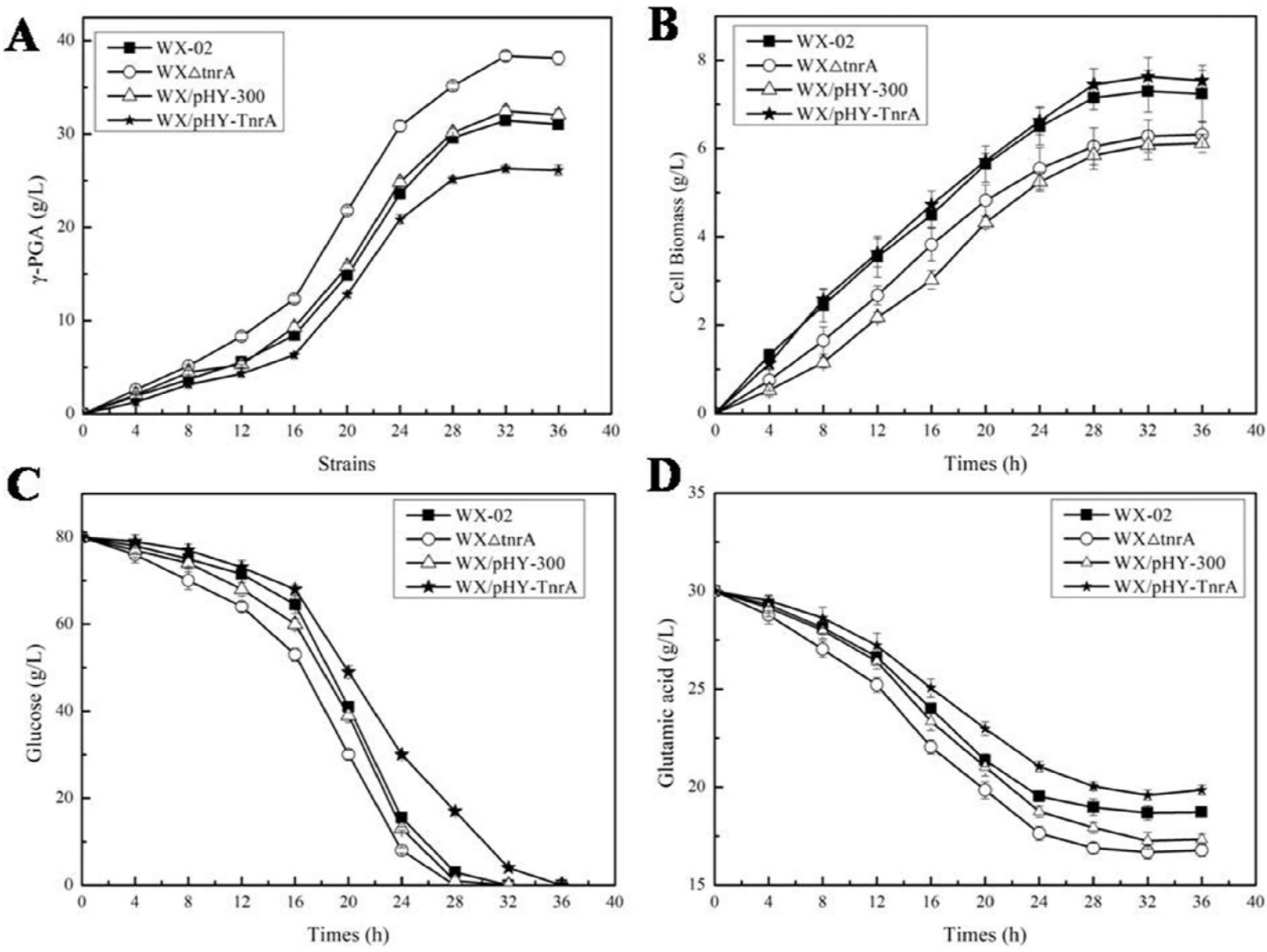
Table 2 The primers used in this research

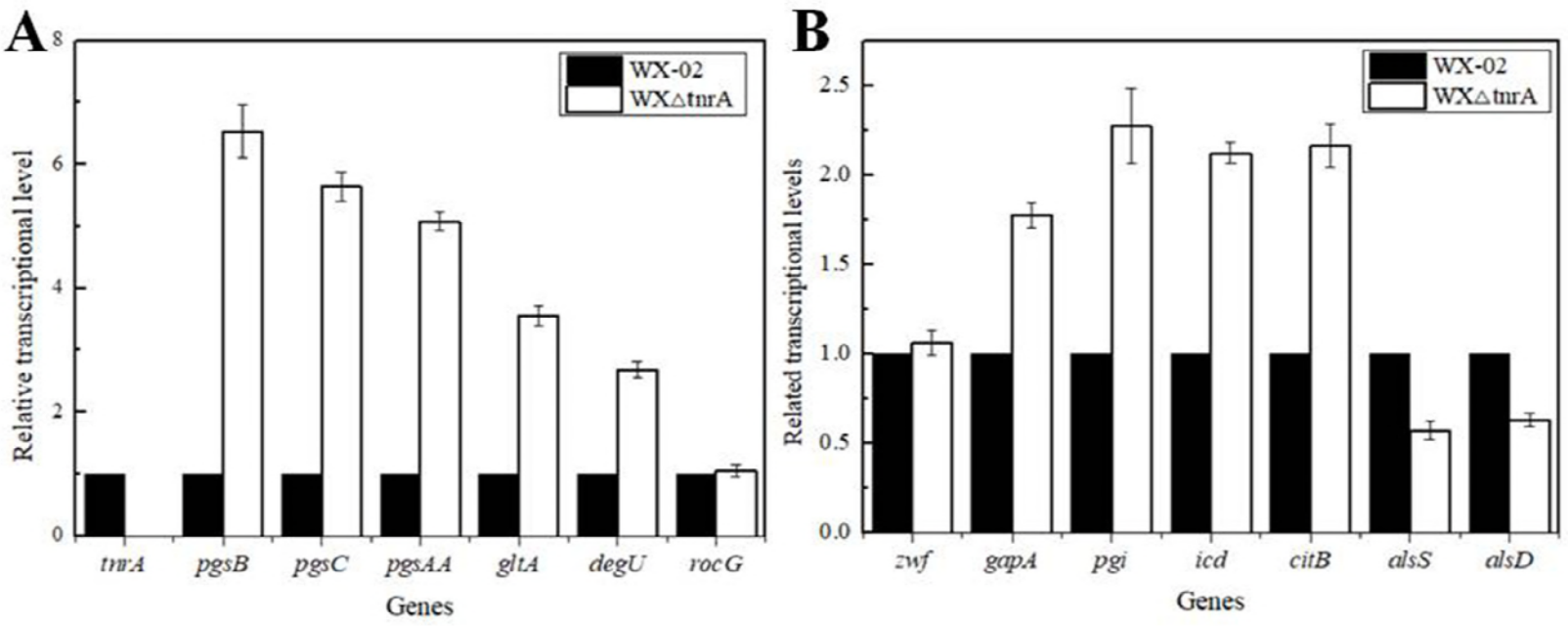
Primers	Sequence 5'-3'	Function
TnrA-F1	GGGAGCTCTTCCCGATTATGCTCACC	Amplication of upstream homologous arm of *tnrA*
TnrA-R1	AATGAGCGTTAAGCTGGCCTTTTTCCCACCCCTAAGATGACTT	
TnrA-F2	AAGTCATCTTAGGGGTGGGAAAAAGGCCAGCTTAACGCTCATT	Amplication of downstream homologous arm of *tnrA*
TnrA-R2	GCTCTAGAGAATGTCCACGAAATGCT	
TnrA-KYF	CATAGCACGGTGAACTTCTT	Verification of *tnrA* mutant strain
TnrA-KYR	GATAAAGAATAAATGATTTCAG	
degU-KFI	CG**GGATCC**CGCGCATCTTCAGAGACAAAGGAACAG	Amplication of upstream homologous arm of *degU*
degU-KRI	CCTGCGTTCTGTCGTTTACATTCATTGACACCTTCACGGAATAACT GATG	
degU-KF2	CATCAGTTATTCCGTGAAGGTGTCAATGAATGTAAACGACAGAAC GCAGG	Amplication of downstream homologous arm of *degU*
degU-KR2	GCTCTAGAGCGTCGTCAACCATAAAATAAGCCCTC	
degU-KYF	GGAAGAAAGAAAAAGGGTCTCAAGG	Verification of *degU* mutant strain
degU-KYR	GAACGATCAGCTTGTTCTCAAAATG	
P43-F	CGGAATTC TGATAGGTGGTATGTTTTCG	Amplication of P43 promoter
P43-R	AAGAAAGATCTTCGACTGTCATGTGTACATTCCTCTCTTACC	
TnrA-F	GGTAAGAGAGGAATGTACACATGACAGTCGAAGATCTTTCTT	Amplication of gene *tnrA*
TnrA-R	GAAATCCGTCCTCTCTGCTCTTTTAACGGTTTTTGTATTTAAAA	
TamyL-F	TTTTAAATACAAAAACCGTTAAAAGAGCAGAGAGGACGGATTTC	Amplication of *amyL* terminator
TamyL-R	GC TCTAGA GCCGCAATAATGCCGTCGCACTG	
PpgsB-F	GATCCTGAATCCATCCTTCAA	Amplication of *pgsB* promoter
PpgsB-R	TAAATGCCGATCCCAACAACG	
PpgsB-1-F	GATCTTTGTCACGGAAAATTTGATCTTTGTCACGGAAAATTT	Amplication of *pgsB* promoter without TnrA binding site
PpgsB-1-R	AAATTTTCCGTGACAAAGATCAAATTTTCCGTGACAAAGATC	
T2-F	ATGTGATAACTCGGCGTA	Verification of *tnrA* knockout vector
T2-R	GCAAGCAGCAGATTACGC	
pHY-F	GTTTATTATCCATACCCTTAC	Verification of expression vector
pHY-R	CAGATTTCGTGATGCTTGTC	
28-TnrA-F	CGGAATTCATGACAGTCGAAGATCTTTCTTA	Amplication of gene *tnrA* for induced expression vector construction
28-TnrA-R	CCGCTCGAGTTAACGGTTTTTGTATTTAAAA	
28-Y-F	TAATACGACTCACTATAGGG	Verification of induced expression vector
28-Y-R	TGCTAGTTATTGCTCAGCGG	

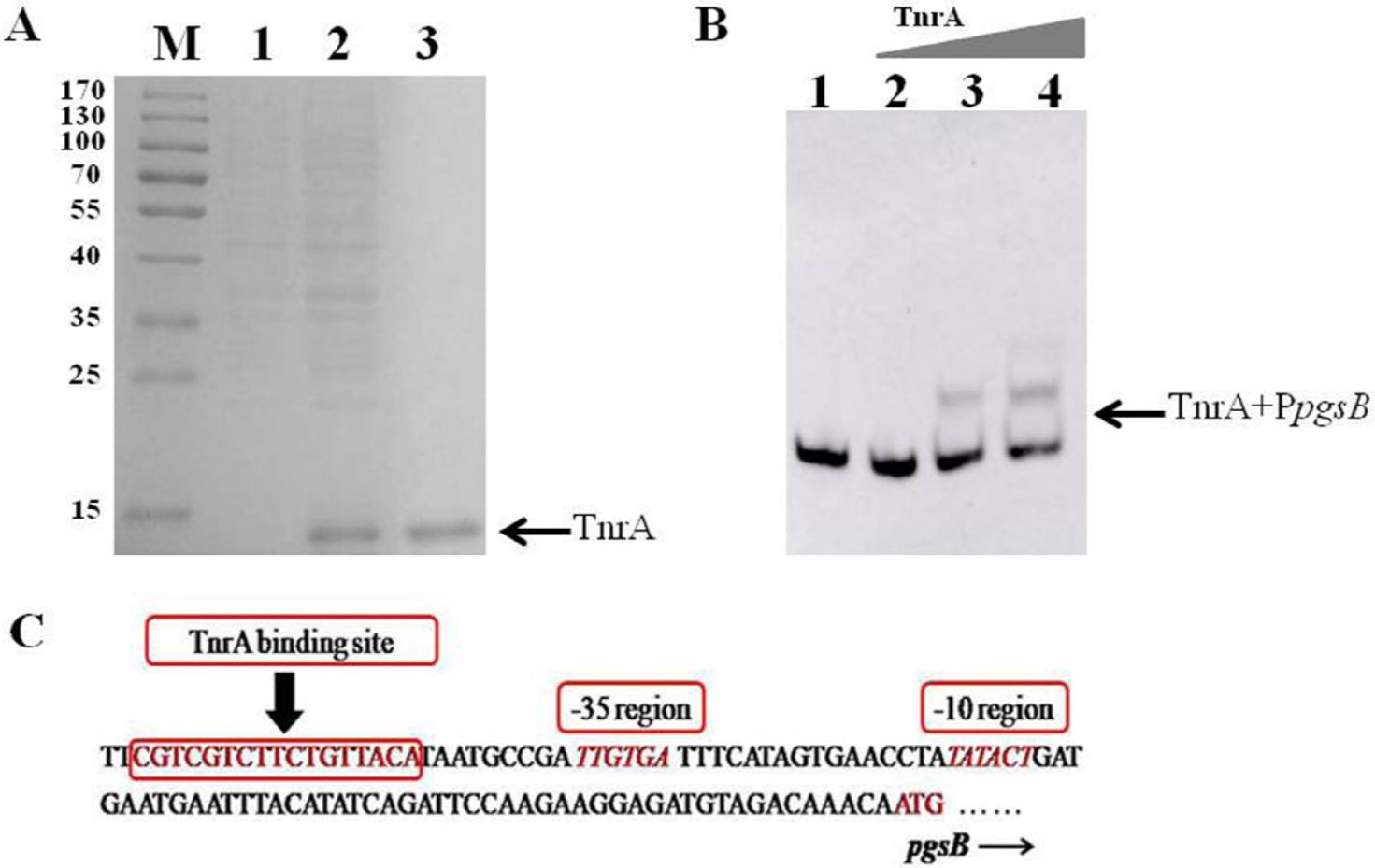
Table 3 The predicted TnrA box of γ-PGA synthase gene *pgsBCAA* in various *Bacillus* strains

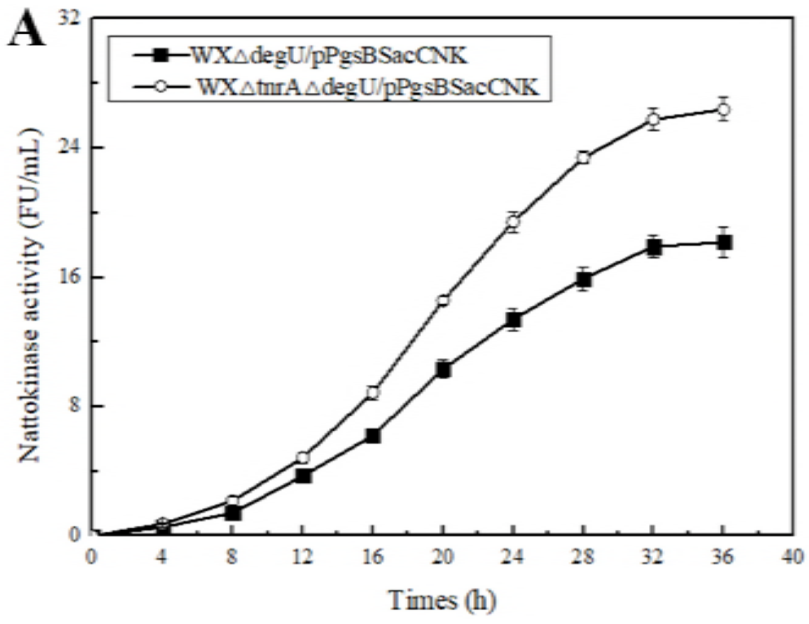
Strains	Genes names	Annotations	TnrA boxs
Bacillus licheniformis WX-02	*pgsBCAA*	γ-PGA synthase	CGTCGTCTTCTGTTACA
Bacillus licheniformis 14580	*pgsBCAA*	γ-PGA synthase	CGTCGTCTTCTGTTACA
Bacillus subtilis 168	*pgsBCAA*	γ-PGA synthase	GGGAAGATTATGTTACA
Bacillus amyloliquefaciens DSM7	*pgsBCAA*	γ-PGA synthase	GAGGAGATTATGTTACA

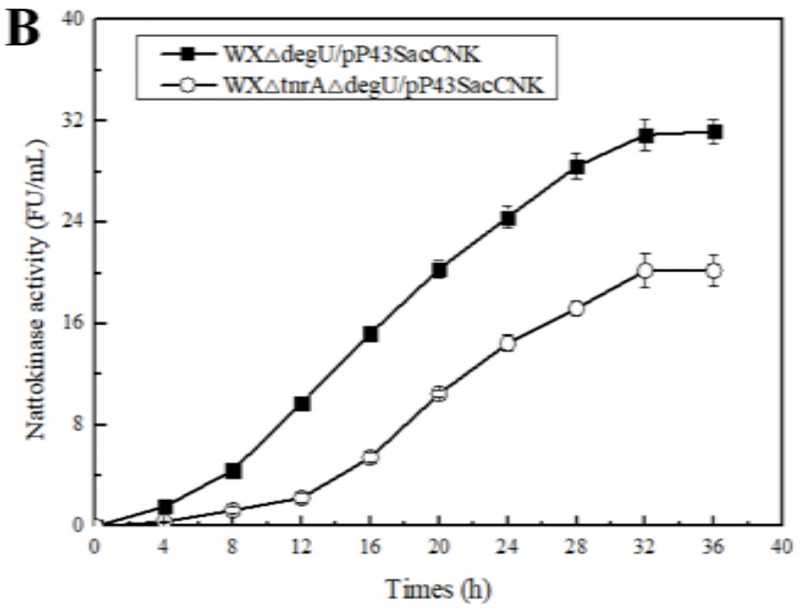

γ-PGA (g/L)
0
10
20
30
40
**
**
WX-02
WXΔtnrA
WX/pHY-300
WX/pHY-TnrA
Strains

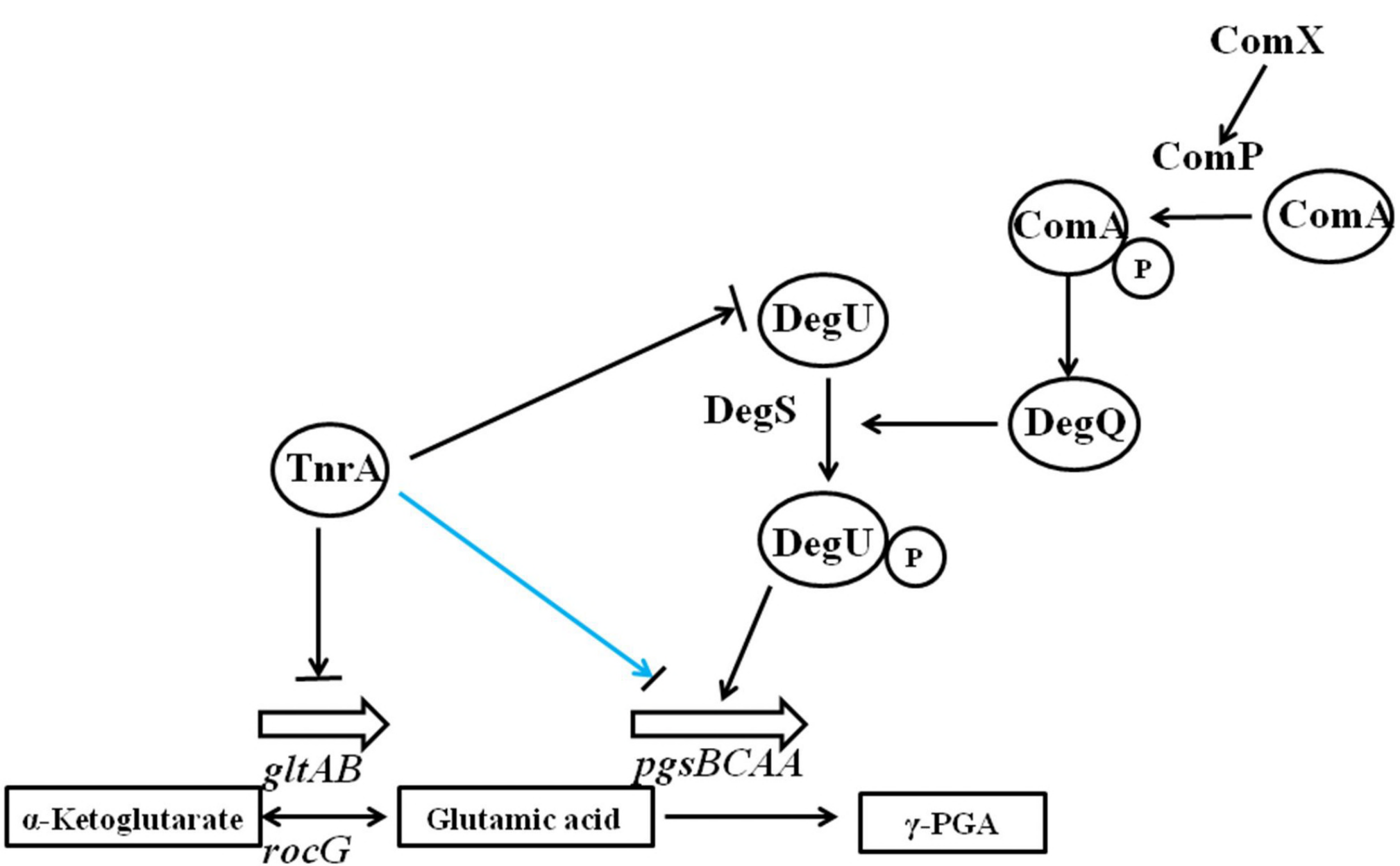

A
WX-02
WXΔtnrA
WX/pHY-300
WX/pHY-TnrA
γ-PGA (g/L)
Strains
B
Cell Biomass (g/L)
Times (h)
C
Glucose (g/L)
Times (h)
D
Glutamic acid (g/L)
Times (h)


A
Relative transcriptional level
0
2
4
6
8
WX-02
WXΔtnrA
tnrA
pgsB
pgsC
pgsAA
gltA
degU
rocG
Genes
B
Related transcriptional levels
0.0
0.5
1.0
1.5
2.0
2.5
WX-02
WXΔtnrA
zwf
gapA
pgi
icd
citB
alsS
alsD
Genes


A
M
1
2
3
170
130
100
70
55
40
35
25
15
TnrA
B
TnrA
1
2
3
4
TnrA+P*pgsB*
C
TnrA binding site
-35 region
-10 region
TTCGTCGTCTTCTGTTACATAATGCCGA TTGTGA TTTCATAGTGAACCTA TATACT GAT
GAATGAATTTACATATCAGATTCCAAGAAGGAGATGTAGACAAACA ATG ……
pgsB


A
WXΔdegU/pPgsBSacCNK
WXΔtnrAΔdegU/pPgsBSacCNK
Nattokinase activity (FU/mL)
Times (h)
0
8
16
24
32
0
4
8
12
16
20
24
28
32
36
40


B
WXΔdegU/pP43SacCNK
WXΔtnrAΔdegU/pP43SacCNK
Nattokinase activity (FU/mL)
Times (h)
0
8
16
24
32
40
0
4
8
12
16
20
24
28
32
36
40


ComX
ComP
ComA
ComA
P
DegU
DegS
DegQ
TnrA
DegU
P
gltAB
pgsBCAA
α-Ketoglutarate
rocG
Glutamic acid
γ-PGA