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Understanding energy fluctuation during the transition state: The role of AbrB in *Bacillus licheniformis*

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Abstract

Background Limited research has been conducted on energy fluctuation during the transition state, despite the critical role of energy supply in microbial physiological metabolism.

Results This study aimed to investigate the regulatory function of transition state transcription factor AbrB on energy metabolism in *Bacillus licheniformis* WX-02. Firstly, the deletion of *abrB* was found to prolong the cell generation time, significantly reducing the intercellular ATP concentration and NADH/NAD^+ ratio at the early stage. Subsequently, various target genes and transcription factors regulated by AbrB were identified through in vitro verification assays. Specifically, AbrB was shown to modulate energy metabolism by directly regulating the expression of genes *pyk* and *pgk* in substrate-level phosphorylation, as well as genes *narK* and *narGHIJ* associated with nitrate respiration. In terms of oxidative phosphorylation, AbrB not only directly regulated ATP generation genes, including *cyd*, *atpB*, *hmp*, *ndh*, *qoxA* and *sdhC*, but also influenced the expression of NAD-dependent enzymes and intracellular NADH/NAD^+ ratio. Additionally, AbrB positively affected the expression of transcription factors CcpN, Fnr, Rex, and ResD involved in energy supply, while negatively affected the regulator CcpA. Overall, this study found that AbrB positively regulates both substrate-level phosphorylation and oxidative phosphorylation, while negatively regulating nitrate respiration.

Conclusions This study proposes a comprehensive regulatory network of AbrB on energy metabolism in *Bacillus*, expanding the understanding of regulatory mechanisms of AbrB and elucidating energy fluctuations during the transition state.

Keywords Transcription factor AbrB, *Bacillus licheniformis*, Energy fluctuation, Energy metabolism, Transcription regulation

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Background

Microbial cells consist of material, energy and information, and their normal life activities cannot be sustained without adequate supplies of these components. Energy is primarily carried by high-energy phosphate compounds, such as adenosine triphosphate (ATP), which plays an essential role in maintaining cellular homeostasis, promoting cell growth, and facilitating metabolite synthesis [1]. As the expands of microbial population, the finite culture ingredients become depleted, leading to a gradual deficiency in resources and an insufficient intracellular energy supply. Analyzing the regulatory network of energy metabolism and understanding the fluctuations in energy during the cultivation process will be beneficial for engineering transformations [2].

Bacteria have developed mechanisms to sense changes in their extracellular environments, which enable them to regulate energy metabolism. Quorum sensing and stress signaling systems mediated by σ factors play crucial roles in this process [3]. Research indicates that genes associated with energy metabolism are regulated by quorum sensing factors released during intercellular communication [4]. The addition of quorum-sensing inhibitor barley base to the medium significantly reduced intracellular levels of cAMP and NAD^+ in *Pseudomonas aeruginosa*, leading to a disruption in energy metabolism [5]. In contrast to quorum sensing system, which coordinates the behaviors of multicellular populations, stress signaling system operates at the single-cell level. Environment-induced σ factors replace the original factors to help bacteria respond to specific environment conditions, adapt to stress, and produce virulence factors [6]. Sigma A is the principal factor mediating constitutive expression of various genes, while another important factor, σ^H, can be induced by pressure, pH, carbon source, and availability of amino acids, and other stimuli, thereby generating a complex regulatory network through other transcription factors [7, 8].

The transition state marks the shift from logarithmic growth to stable growth, and regulatory changes occurring during this period are critically important for physiological metabolism [9]. AbrB is a pivotal global transcription factor in the transitional phase, initially investigated in *Bacillus subtilis* [10]. The gene coding AbrB is activated by σ^A and inhibited by cascaded regulation of SigH-Spo0A-AbrB [11, 12]. The master regulator of sporulation, Spo0A, binds to and directly represses the *abrB* promoter [13]. Furthermore, an *abrB* mutation has been shown to restore biofilm formation to a *spo0A* mutant strain [14]. Sigma-H may activate the expression of the *spo0A* gene, thereby indirectly repressing AbrB expression [15]. Moreover, AbrB can perform a global regulatory function, exerting a negative regulatory effect on various genes, including sporulation gene *spo0E* [16], alkaline protease gene *aprE* [17], entianin synthesis gene clusters *etnBTC* [18], surfactin-forming gene operon *srfABC* [19] and pulcherriminic acid synthetase gene cluster *yvmC-cypX* [20]. It is noteworthy that most of these genes are involved in the production of middle and late secondary metabolites. However, limited research has investigated the AbrB's role in regulating energy metabolism in *Bacillus subtilis*, with existing studies primarily relying on omics data. Results from ChIP-Chip and DNA microarrays analyses suggested that several respiratory chain genes, such as *cydABC*, *ctaBCDEFG* and *qcrABC*, may be regulated by AbrB [9, 21], though the regulation model is not entirely consistent. Therefore, the specific regulatory mechanisms by which AbrB influences energy metabolism warrant further investigation.

Bacillus licheniformis is a significant industrial microorganism acclaimed for its excellent secretion performance and production capabilities. These attributes have enabled its successful application in the efficient production of various chemicals [22, 23] and industrial enzymes [24, 25]. Energy supply primarily involves substrate-level phosphorylation, oxidative phosphorylation, and nitrate respiration; however, the transcriptional regulation of main energy production remains inadequately explored. This research aims to establish a regulation network of AbrB on energy metabolism in *B. licheniformis*, which not only improve the understanding of energy fluctuations during the transition state, but also provide a foundation for energy metabolic engineering to improve target product production.

Materials and methods

Strains, plasmids and cultivation conditions

The strains and plasmids used in this study were listed in Supplementary Tables 1, and primers for PCR amplification were shown in Supplementary Table 2. *Escherichia coli* DH5α was used for vector construction, *E. coli* BL21(DE3) was applied for AbrB protein induction expression, and *B. licheniformis* WX-02 was served as the original strain for constructing recombinant strains. The plasmid T_2(2)-Ori was applied for gene deletion vector construction, and pET28a was used for constructing protein induction expression vector. Luria-Berteni (LB) medium with or without antibiotic (20 μg/mL kanamycin or tetracycline) was served as the basal cultivation medium. The M9 medium contained 10 g/L Glucose, 15 g/L $Na_2HPO_4{\cdot}12H_2O$, 3.0 g/L KH_2PO_4, 0.5 g/L NaCl, 1.0 g/L NH_4Cl, 0.492 g/L $MgSO_4{\cdot}7H_2O$, 0.011 g/L $CaCl_2$, pH 7.0. For physiological parameter detection and RNA preparation, the seeds were cultivated in 5 mL LB broth for 16 h, and then transferred into M9 medium with 3% inoculum. All strains were cultivated at 37 °C, except for strain subcultures and protein induction.

Construction of gene *abrB* deletion strain

Temperature-sensitive plasmid T_2(2)-Ori was used for gene *abrB* deletion in *B. licheniformis*. Firstly, the upstream and downstream homology arms of *abrB* were respectively amplified by corresponding primers (abrB-KF1/KR1 and abrB-KF2/KR2), then ligated through Splicing Overlap Extension (SOE)-PCR, and assembled into T_2(2)-Ori using 2X MultiF Seamless Assembly Mix (RK21020, ABclonal Technology Co., Ltd.). Subsequently, the products were transformed into *E. coli* DH5α, and the transformat was verified by colony PCR using primers T2-L and T2-R, as well as DNA sequence (Sangon Biotech, China). The resultant *abrB* knockout plasmid was designated as T_2-△abrB.

Then, plasmid T_2-△abrB was electro-transferred into *B. licheniformis* WX-02, and positive clones were cultivated in LB medium with 20 μg/mL kanamycin at 45 °C, and then sub-cultured three times to obtain the single-crossover recombinants. Subsequently, the positive recombinants were cultivated in LB medium at 37 °C for multiple generations to shed the plasmid, and *abrB* deletion strain was obtained by colony PCR and DNA sequence, named as WX-02△abrB.

Construction of gene overexpression strain

The method for constructing the gene overexpression strains were referred to our previously reported research [26, 27]. To construct WX-02/pHY-abrB, the gene *abrB* along with its promoter was amplified from *B. licheniformis* WX-02 genome by PCR using primers pHY-abrB-F and pHY-abrB-R. The plasmid pHY300PLK harboring terminator TamyL was amplified by PCR using primers 300-T5-F and 300-T5-R, and PCR product was applied as the vector backbone. Gene *abrB* and corresponding plasmid vector backbone were connected using 2X MultiF Seamless Assembly Mix (RK21020, ABclonal Technology Co., Ltd.), resulting in the recombinant plasmid pHY-abrB. Plasmid pHY-abrB was then electroporated into *B. licheniformis* WX-02 to obtain AbrB expression strain WX-02/pHY-abrB.

For the pHY-abrB-PA plasmid, the upstream arm was amplified using primers pHY-abrB-F and AbrB-PA-AR, while the downstream arm was obtained using primers pHY-abrB-R and AbrB-PA-BF. Subsequently, the upstream and downstream homologous arms were connected via SOE PCR, and the vector pHY-abrB-PA and strain WX-02/pHY-abrB-PA were obtained successively according to the WX-02/pHY-abrB construction method described above.

AbrB induction expression and purification

The plasmid pET28a(+) (Novagen, Denmark) was applied for AbrB protein induction expression. Gene *abrB* from *B. licheniformis* was amplified and inserted into pET28a using 2X MultiF Seamless Assembly Mix, and then transferred into *E. coli* BL21(DE3). After screening with kanamycin resistant, the positive clones were verified by PCR and DNA sequence, and AbrB induction expression strain was denoted as *E. coli* BL21/pET28a-abrB.

To obtain AbrB protein, BL21/pET28a-abrB was cultivated in 5 mL LB medium with 20 μg/mL kanamycin for 12 h, and transferred into 50 mL LB medium with a 2% inoculation. Then, 0.3 mM IPTG were added to induce AbrB protein expression until OD_{600} reached 0.6–0.8, and cultivated at 16 °C for 8–14 h. The collected cells were lysed using a pressure cell disruptor (JN-02C, Jnbio, China), and supernatant was purified and collected with Ni NTA beads with its gravity Column (Smart-lifesciences, China), and the resulting solution was dialyzed for 2 days to obtain the purer protein. Finally, the purified AbrB protein was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Electrophoretic mobility shift assay

Probe DNA targeting promoter regions of different genes were amplified by 5'-biotin labeled primers (synthesized by Sangon Biotech, China). Following amplification, these DNA probes were extracted and purified using E.Z.N.A. Gel Extraction Kit D2500-02 (Omega Bio-Tek). In the subsequent EMSA assays, Chemiluminescence EMSA Kit GS009 (Beyotime, China) was used, and the amount of DNA probe added in all EMSA binding reactions was 10 ng, and each group included a negative control without protein addition (lane 1) and sample reactions with different concentrations of AbrB protein (lane 2 and 3), and the final image was performed by Amersham Imager 600 (Gelifesciences, USA). The EMSA condition optimization of the experiment was referred to Furbass et al. [28], and the results of reaction condition optimization and control test were shown in Supplementary Table 2.

Analytical methods

Due to the large amount of flocculent precipitate produced by gene *abrB* deletion strain at the early stage of cell growth, the cell biomass measured in this study was the cell turbidity after removal of precipitation, which was represented as the absorbance value at 600 nm (OD_{600}). Cell generation time was performed according to the method recorded by Bachmann et al. [29]. The difference is that cell turbidity values (OD_{600}) are used instead of colony forming units (CFU). According to the growth curve, the time when the cells reached the stable stage was determined as the subculture time (that is, a growth cycle). The initial cell turbidity when the strain was added to the fresh liquid medium was denoted as N0, and the cell turbidity at the stable stage was denoted as Nf. The population doubling number in one growth cycle was calculated by the formula $\log_2 100$ (Nf/N0). Cell

generation G is the number of cell multiplications in one hour.

ATP concentration was measured by using ATP Assay Kit S0026 (Beyotime, Shanghai, China), as previously reported [30]. Meanwhile, luminescence values (RLU) at 550 nm were determined using SpectraMax iD3 Multi-Mode Microplate Readers (Molecular Devices, USA). The final ATP concentration of unit cell was the ratio of ATP content to the corresponding biomass. The total concentration of NAD^+ and NADH (expressed as NAD_{total}), and NADH concentrations were determined using NAD^+/NADH Assay Kit (Beyotime, Shanghai, China), and NAD^+ concentration and NADH/NAD^+ ratio were calculated based on the above detection results. Nitrate concentration was detected by salicylic acid method, and nitrite concentration was determined by naphthalamine colorimetric method, as previously reported [31].

For gene transcriptional level analysis, the strain was cultured in the M9 medium for 8–9 h (pre-logarithmic phase), and total RNA was extracted using RNA extraction kit (FORE GENE, China), and reverse transcriptional reaction was conducted by using ABScript III RT Master Mix for qPCR with gDNA Remover RK20429 (ABclonal Technology, China). The real-time PCR was performed by using AceQ qPCR SYBR Green Master Mix (Nanjing Vazyme Biotech Co., Ltd.), and *16 S rDNA* was served as the reference gene. The gene transcription level of control strain WX-02 was set as a constant value of 1.000, against which the relative transcription level of target gene in recombinant strain was calculated as a ratio, which is determined using the $2^{\wedge-\triangle\triangle Ct}$ method [32]. The significance of difference at gene transcription level was established, when the relative transcription level exceeds 2 or falls below 0.5.

Computational analysis

The MEME tools (http://meme-suite.org/) were used to recognize AbrB binding sequences in the upstream region of target genes in *B. licheniformis*, and the -10 or -35 region of promoter was predicated by BPROM tool (http://www.softberry.com/berry.phtml). The distribut ion of AbrB binding sequences on the target genes was mapped using SnapGene software.

Data analysis

At least three biological replicates were performed for each experiment. The *t* test was applied to compare the mean values using SPSS 18.0 and all data were conducted to analyze the variance at $P<0.05$ (*) and $P<0.01$ (**), $p<0.05$ and fold-change < 0.5 or > 2 (#).

Results

AbrB plays as an important role in energy metabolism of *B. licheniformis*

Firstly, gene *abrB* deletion strain WX-02△abrB and overexpression strain WX-02/pHY-abrB (harboring its native promoter) were constructed. Given that phosphorylated Spo0A binds to the promoter of gene *abrB* and inhibits its expression, the Spo0A binding site in *abrB* promoter was deleted to obtain strain WX-02/pHY-abrB-PA.

To investigate the relationship between *abrB* gene expression and cell growth, the relative transcription level and the optical density at 600 nm (OD_{600}) of these aforementioned strains were measured. As illustrated in Fig. 1A, after *abrB* deletion, *abrB* gene was hardly expressed in the WX-02 strain. In terms of the growth curve, WX-02 experienced a lag phage from 0 to 2 h, reaching a stable growth phase at 12 h, with the most pronounced difference occurring at 8 h, where the OD_{600} of WX-02△abrB (1.598) was 45.2% lower than that of WX-02 (2.915) ($P<0.01$). Utilizing the method described by Bachmann et al. (2012), the cell generation time of WX-02 was calculated to be 3.01 h. In contrast, WX-02△abrB entered the stable phase at 14 h, exhibiting a longer cell generation time of approximately 3.35 h. Furthermore, the transcription levels of *abrB* gene were significantly increased in pHY-abrB and pHY-abrB-PA free expression strains. There was no significant difference in cell biomass between WX-02/pHY-abrB and control strain WX-02/pHY. However, WX-02/pHY-abrB-PA showed higher cell biomass, particularly after 10 h (Fig. 1B). The cell generation time for WX-02/pHY-abrB was 3.56 h, slightly longer than that of WX-02/pHY (3.43 h), while WX-02/pHY-abrB-PA had a generation time of 3.30 h, representing a reduction of 0.26 h compared to WX-02/pHY. Therefore, the absence of *abrB* resulted in the prolonged cell generation, decreased biomass, whereas AbrB overexpression produced the opposite effect, suggested that transcriptional factor AbrB promoted cell growth during the logarithmic phase.

Furthermore, to explore the function role of *abrB* on energy metabolism, the intracellular concentrations of ATP and NAD were measured during the logarithmic phase. Strain WX-02△abrB exhibited a significantly lower ATP concentration of 0.275 μM ATP per OD_{600}, decreased by 51.84% compared to WX-02(0.571 μM ATP per OD_{600}) ($P<0.01$). Conoversely, *abrB* overexpression led to an increase of intracellular ATP content (Fig. 1C, Supplementary Table 3), indicated that AbrB may influence the intracellular energy supply. Besides, the total concentration of NAD^+ and NADH in WX-02△abrB was appoximately 1.44 times higher than that of WX-02, while the NADH/NAD^+ ratio in WX-02△abrB was decreased by 46.4%, compared to WX-02 (Fig. 1D, Supplementary Table 3). The NAD concentrations in WX-02/

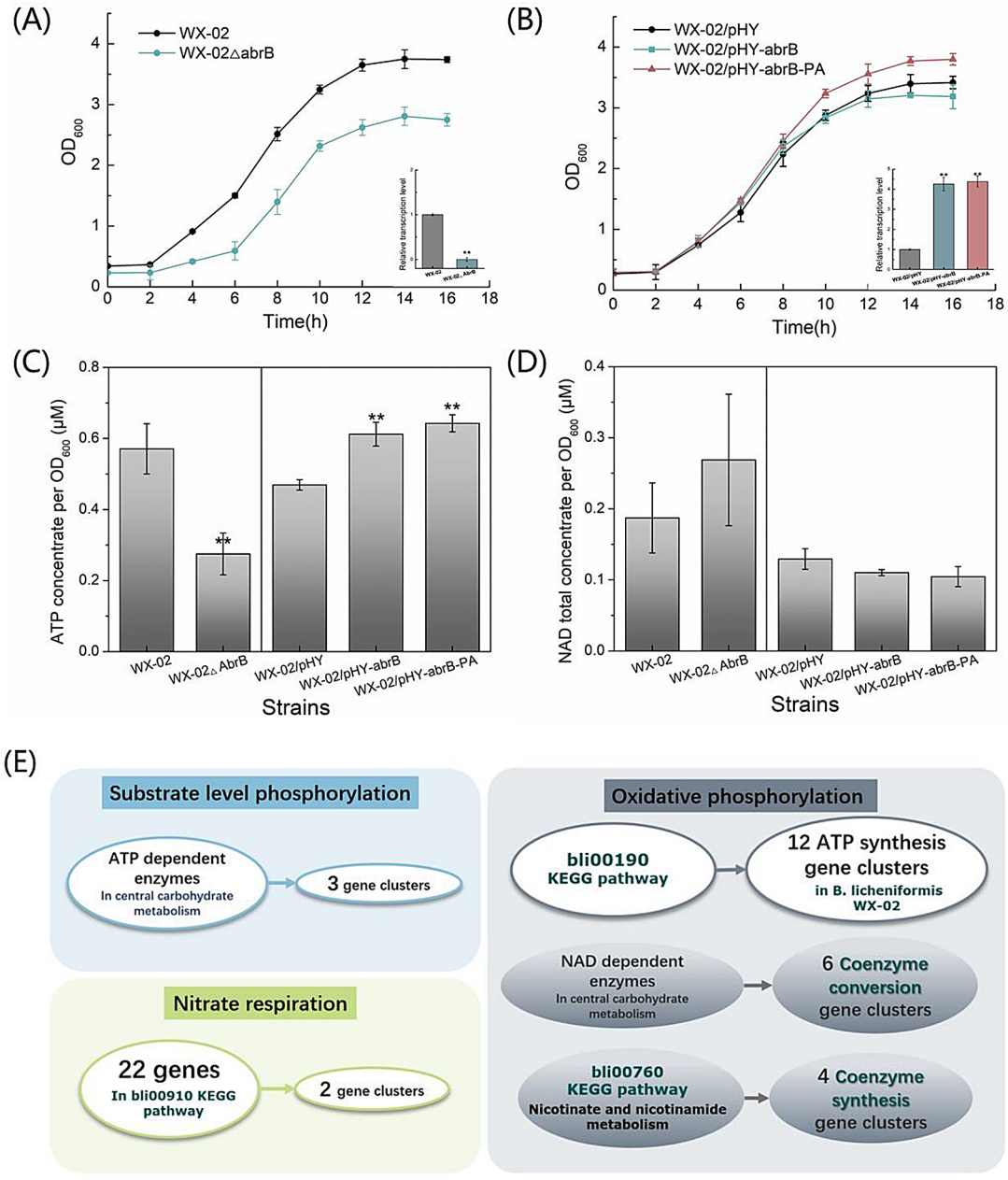


Fig. 1 Physiological parameter detection of AbrB deletion and over-expression strains. **A** Growth curves and relative transcription levels of *abrB* in WX-02 and WX-02△abrB. **B** Growth curves and relative transcription levels of *abrB* in WX-02/pHY, WX-02/pHY-abrB and WX-02/pHY-abrB-PA. **C** ATP concentration per OD_{600}. **D** NAD total concentration per OD_{600}. **E** Mining of target genes and transcription factors in energy metabolism that regulated by AbrB

pHY-abrB and WX-02/pHY-abrB-PA were slightly lower than that of WX-02/pHY, with their NADH/NAD^+ ratios were increased by 76.1% and 105.6%, respectively. These results suggested that AbrB could influence intracellular ATP concentration and NADH/NAD^+ ratio during the logarithmic phase. Additionally, cell growth slowed after the expression of pHY300PLK, which may be the primary reason for the relative decrease in NAD content per unit cell during the logarithmic prophase.

Mining of target genes and transcription factors regulated by AbrB

Combining the metabolic pathways in KEGG (Kyoto Encyclopedia of Genes and Genomes) and genetic information of NCBI database (National Center for Biotechnology Information) of *B. licheniformis* 14,580, which has 99.91% sequence similarity to *B. licheniformis* WX-02, we identified 31 genes or gene clusters in energy metabolism. These genes are categorized into three modules: substrate-level phosphorylation, oxidative phosphorylation

and nitrate respiration (Fig. 1E). In the substrate-level phosphorylation module, three genes (*pgk*, *pyk*, *sucC*) that encode ATP-dependent enzymes involved in central carbohydrate metabolism, and 12 genes or gene clusters associated with respiratory electron transport chain in oxidative phosphorylation module were selected, basing on KEGG pathway of bil00190. Additionally, given that the content and proportion of intracellular cofactors changed following *abrB* deletion, 11 cofactor-dependent dehydrogenases genes and 4 cofactor generation gene clusters were identified. In the nitrate respiration module, we highlighted the nitrate reductase gene cluster *narGHIJ* and nitrite transportation gene *narK*, as referenced in KEGG pathway bli00910. Furthermore, 7 potential transcription factors were discovered through the literature review (Supplementary Table 4).

Analysis of AbrB transcriptomics data suggests that the indirect regulation mediated by other transcription factors may significantly influence the middle and late stage of cell growth, potentially complicating the identification of direct target genes [33]. Therefore, to uncover additional genes directly regulated by AbrB, we explored transcriptional changes during the pre-logarithmic phase in subsequent research.

Positive regulation mediated by AbrB on substrate-level phosphorylation

As for substrate-level phosphorylation, the transcription levels of gene *sucC* in TCA cycle did not show significant changes in WX-02△abrB, while the transcription levels of genes *pgk* and *pyk* in EMP pathway were significantly reduced to 0.49 and 0.41, respectively, compared to WX-02 (Fig. 2A). Subsequently, to verify in vivo whether *sucC*, *pgk* and *pyk* genes are affected by AbrB, we attempted to introduce their promoter-mediated target protein expression vectors into both WX-02 and WX-02△abrB, respectively. However, due to the extremely low conversion efficiency of *abrB*-deficient strains, the above validation experiments could not be achieved.

Subsequently, electrophoretic mobility shift assays (EMSA) were performed to verify the direct interaction between AbrB with genes *pgk* and *pyk*. The addition of AbrB at a concentration of 20 μM resulted in significant shifts in the EMSAs for both the *pyk* and *pgk* promoters (lanes 2 and 3 in Fig. 2B-C). Importantly, this combination exhibited good specificity, as it was unaffected by the addition of non-specific competing DNA (lanes 4). These results suggested that AbrB could directly bind to the promoter regions of *pgk* and *pyk*, thereby positively regulated their expression. Besides, the interactions of AbrB with the carbon metabolism transcription factor genes *ccpC* and *ccpN* were assessed (Fig. 2A), and the relative

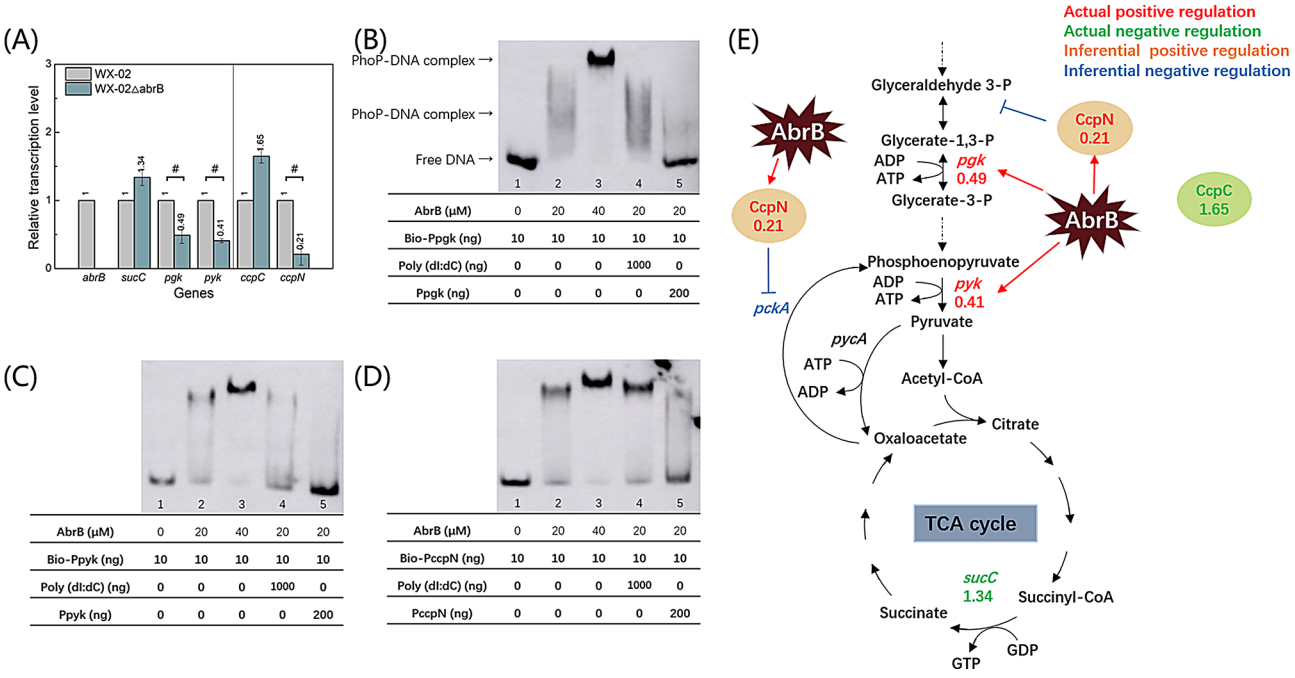


Fig. 2 Positive regulation of AbrB on substrate level phosphorylation. **A** Relative transcription levels of substrate level phosphorylation genes at 8–9 h of cultivation. **B** EMSA of AbrB with Ppgk (-252 to + 118). **C** EMSA of AbrB with Ppyk (-282 to + 88). **D** EMSA of AbrB with PccpN (-322 to + 48). Lane 1, no protein; lane 2, 20 μM AbrB; lane 3, 40 μM AbrB; lane 4, 20 μM AbrB and 1 μg non-specific competitive DNA poly(dI: dC); lane 5, 20 μM AbrB and 200 ng DNA probe without biotin label. **E** Regulation map of substrate level phosphorylation by AbrB

transcription level of *ccpC* (1.65) increased insignificantly, while *ccpN* (0.21) exhibited a significant decrease. Subsequent EMSA results displayed a distinct shift after adding 20 and 40 μM Bio-PccpN probes with AbrB (lanes 2 and 3 in Fig. 2D), indicated that AbrB directly regulated *ccpN* expression.

Previous reports have suggested that transcription repressor CcpN negatively regulates the phosphoenolpyruvate carboxykinase gene *pckA* in the gluconeogenesis pathway and glyceraldehyde-3-phosphate dehydrogenase gene *gapB* in *B. subtilis* [34, 35]. After analyzing their sequences for high similarity to those in *B. licheniformis* WX-02, we speculated that AbrB indirectly negatively regulated *pckA* and *gapB* through CcpN. Furthermore, our study also indicated that AbrB directly positively regulated *pyk* and *pgk*, with the regulation pattern of AbrB on substrate-level phosphorylation was illustrated in Fig. 2E.

Positive regulation of AbrB on oxidative phosphorylation

As for the oxidative phosphorylation module, 12 respiratory electron transport chain genes, 11 cofactor-dependent dehydrogenase genes, 3 cofactor generation genes or gene clusters, and 4 transcriptional factor encoding genes were selected as the candidates. Among these, transcriptional levels of respiratory electron transport chain genes (*ndh*, *sdhC*, *qoxA*, *cydA*, *atpB*, and *hmp*), and cofactor-dependent dehydrogenase genes (*icd*, *mdh*, *gapA*, *gndA*, *pdhA*, *zwf*, and *sdhC*) were significantly decreased in WX-02△*abrB*, compared to control strain (Fig. 3A). Conversely, genes *ndhF* (35.16), *ythA* (56.64), *acoA* (43.13), *gapB* (27.81), and *lpdV* (2.75) showed remarkably increased, while no significant change was performed for genes associated with cofactor generation (Fig. 3A). These results suggested that AbrB may play a positive regulatory role in the respiratory chain and cofactor conversion during the early growth stage.

Subsequently, the target genes that performed significant changes in transcription levels were selected for EMSA assays. Given that RNA abundance of *ndhF* and *ythA* was very low in omics data [33], further investigation about these genes was not pursed. EMSA results revealed that all these probes displayed significant shifts (Fig. 3B-C), which suggested that AbrB positively regulated the respiratory electron transport chain genes *cydA*, *atpB*, *hmp*, *ndh*, *qoxA*, and *sdhC*, as well as cofactor conversion genes *mdh*, *gapA*, and *pdhA*, while negatively regulated genes *acoA*, *gapB*, and *lpdV*.

Variations in the regulation patterns of AbrB for NADH-dependent dehydrogenases may be associated with differences in metabolic pathways. Specifically, both genes, *gapA* and *gapB*, encode co-functional aldehyde-3-phosphate dehydrogenase. Previous studies have revealed that *gapA* primarily participates in the glycolytic pathway, whereas *gapB* served as an essential function in gluconeogenic pathway of *E. coli* and *Staphylococcus aureus* [36, 37]. In this study, these two genes were regulated by AbrB in opposite mode, indicated that AbrB promoted glycolysis and inhibited gluconeogenesis. Moreover, the results of residual glucose concentration analysis provided the additional support for the positive regulation of AbrB on carbon metabolism, as the parameters of *abrB* deletion strain were significantly lower than those of control (Supplementary Fig. 2).

To further analyze the indirect regulatory mechanism of AbrB on energy metabolism, four transcription factors, Rex, ResD, NadR, and CcpA, were identified. Among these, the redox regulator Rex has been reported to negatively regulate electron transport chain genes *cydABCD* and NADH dehydrogenase gene cluster *yjiD-ndh* [38, 39]. ResD has been found to positively regulate flavin protein coding gene *hmp* in *B. subtilis*, as well as respiration chain genes *ctaBCDEFG*, *cydABCD*, and *qcrABC* [40, 41]. NadR, a key transcription factor involved in cofactor *de novo* synthesis, owns the capability to inhibit the expression of NAD synthetase gene cluster *nadBAC* [42]. CcpA negatively regulates respiratory chain gene *cydABCD* and cofactor-dependent dehydrogenase genes *acoABCL*, *mdh*, and *icd* [41]. Our results indicated that transcription levels of genes *rex* and *resD* were decreased by 47% and 46%, respectively, in the WX-02△abrB strain. These reductions closely approach our threshold for significant change, defined as a relative transcription level exceeding 2 or falling below 0.5. (Fig. 3A). Besides, consistant with the previous regulatory pattern of cofactor generation genes, transcriptional level of *nadR* did not exhibit significant changes. Notably, transcription level of gene *ccpA* showed a substantial increase in *abrB* deletion strain, rising by 3.71 times compared to WX-02 (Fig. 3A). Subsequently, in vitro interactions were verified, and EMSA results revealed the observable mobility shifts with gradually increasing probe concentrations (Fig. 3C), suggested that AbrB could directly bind to the transcription factor genes *rex*, *resD*, and *ccpA*, positively regulated *rex* and *resD*, while negatively regulated *ccpA*.

Based on the previous studies of these transcription factors in *Bacillus* and high degree of similarity after sequence alignment, it is reasonable to infer the indirect regulation pathways through which AbrB influences energy metabolism in *B. licheniformis*. For instance, AbrB could indirectly exert a negative regulation on *cydABCD* and *yjiD-ndh* operons via Rex, which seems to be inconsistent with the down-regulation of *ndh* gene expression in WX-02△abrB, this discrepancy may be attributed to spatiotemporal regulatory mechanisms. Besides, AbrB indirectly positively regulated *ctaBCDEFG*, *cydABCD* and *qcrABC* through ResD, moreover, it positively regulated respiratory chain gene *cydABCD* and

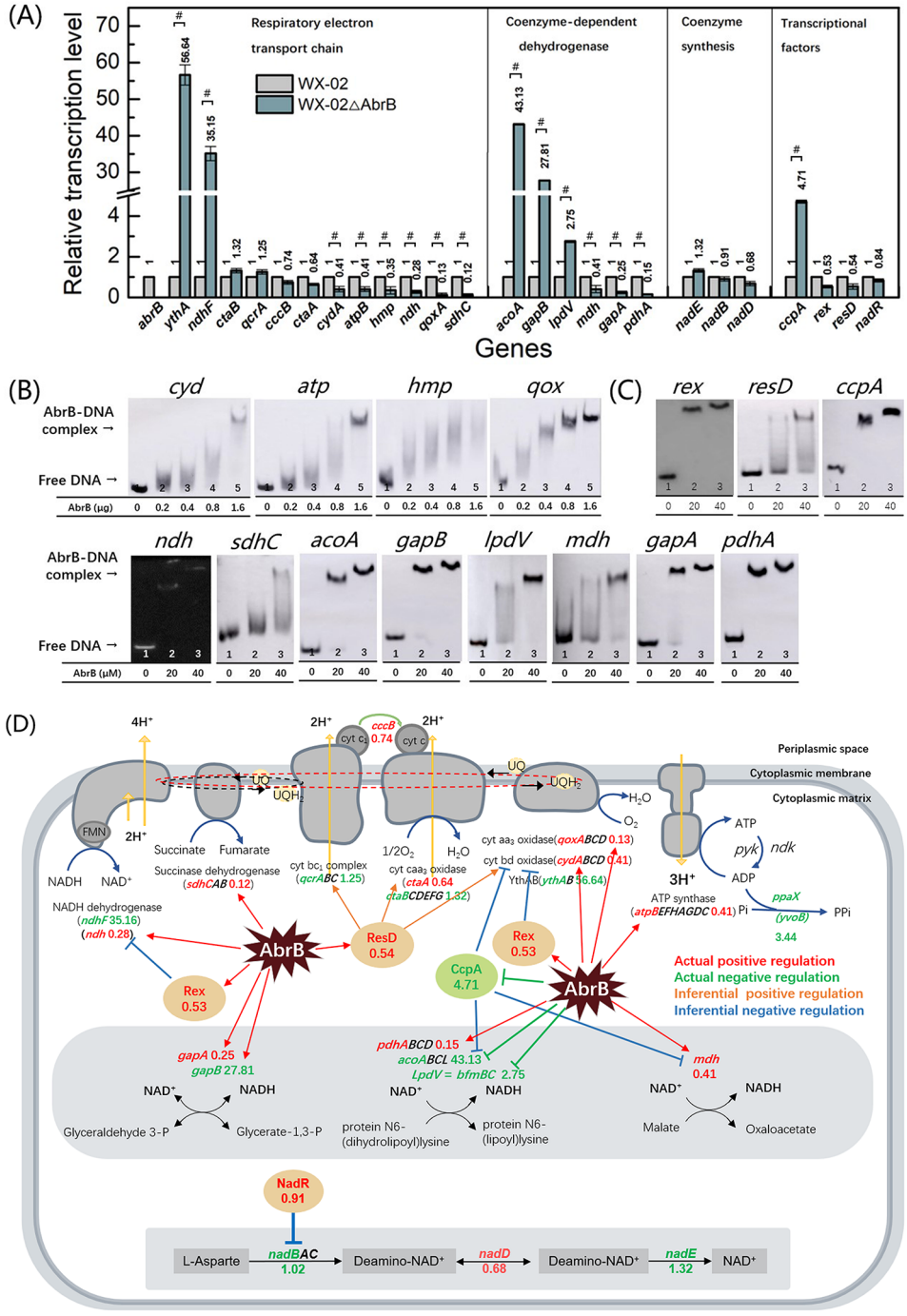


Fig. 3 Positive regulation of AbrB on oxidative phosphorylation. **A** Relative transcription levels of oxidative phosphorylation genes at 8–9 h of cultivation. **B** EMSA of AbrB with oxidative phosphorylation genes. Pcyd (-221 to + 65), Patp (-54 to + 206), Phmp (-154 to + 112), Pqox (-182 to + 31), Pndh (-161 to + 74), PsdhC (-98 to + 310), PacoA (-97 to + 209), PgapB (-189 to + 157), PlpdV (-177 to + 259), Pmdh (-193 to + 71), PgapA (-184 to + 116), PpdhA (-225 to + 135). **C** EMSA of AbrB with oxidative phosphorylation related transcriptional factors. Prex (-54 to + 187), PresD (-116 to + 64), PccpA (-253 to + 192). **D** Regulation map of oxidative phosphorylation mediated by AbrB

cofactor-dependent dehydrogenase genes *acoABCL*, *mdh*, and *icd* by negatively regulating regulator CcpA. Finally, a theoretical regulatory model diagram of AbrB on oxidative phosphorylation was proposed in Fig. 3D.

Negative regulation of nitrate respiration by AbrB

For nitrate respiration, we assessed the relative transcription levels of genes *narGHIJ*, *narK*, and anaerobic transcription factor gene *fnr*. As illustrated in Fig. 4A, the relative transcription levels of *narK* (23.69) and *narGHIJ*

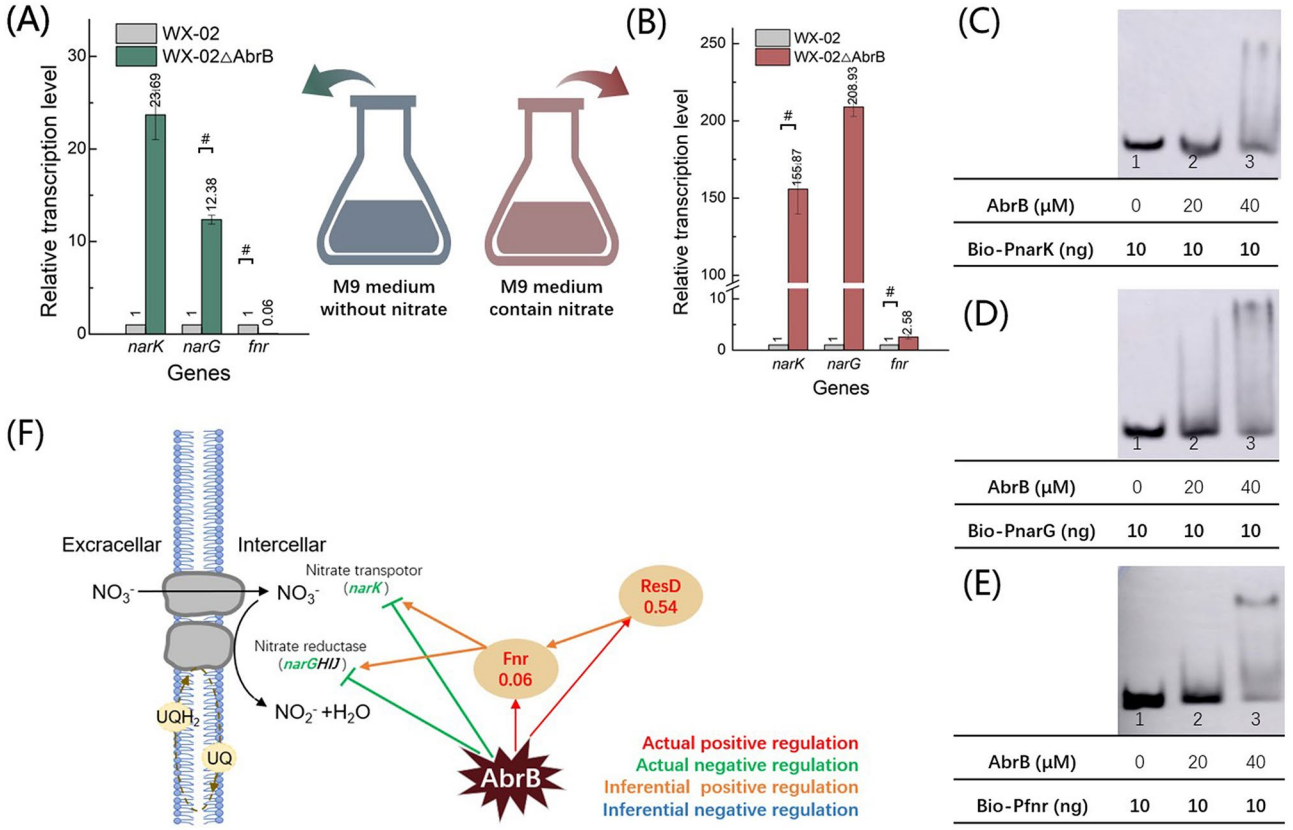


Fig. 4 Negative regulation of AbrB on nitrate respiration. **A** Relative transcription levels of nitrate respiration genes at 8–9 h of cultivation. **B** Relative transcription levels in M9 medium with nitrate addition at 8–9 h of cultivation. **C** EMSA of AbrB with PnarK (-253 to + 219). **D** EMSA of AbrB with PnarG (-200 to + 103). **E** EMSA of AbrB with Pfnr (-259 to + 47). **F** Transcriptional regulation map of nitrate respiration mediated by AbrB

(12.38) were significantly increased in WX-02△abrB, while *fnr* showed a notable reduction to 0.06. This observation suggested a negative regulatory trend for nitrate respiratory genes, contrasting with the positive regulatory pattern of substrate level phosphorylation and oxidative phosphorylation mediated by AbrB.

To further explore the regulation effect of AbrB on nitrate metabolism, sodium nitrate was supplemented into M9 medium to measure OD_{600}, as well as nitrate and nitrite concentrations. According to the results presented in Supplementary Fig. 3, the OD_{600} of *abrB*-deficient strain was significantly lower than that of WX-02 in M9 medium with nitrate addition. Importantly, the nitrate utilization capability of WX-02△AbrB was markedly enhanced compared to control, resulting in a rapid decrease in nitrate content within 6–9 h. The nitrate absorption rate per OD_{600} was measured at 0.035 mg/mL/h, which is 3.39 times greater than that of WX-02. Additionally, the nitrite net generation rate per OD_{600} for WX-02△AbrB (0.157 μg/mL/h) was significantly higher than that of WX-02 (0.006 μg/mL/h), thereby reinforcing the inhibitory effect of AbrB on nitrate utilization.

Subsequently, we re-evaluated the transcription levels of genes *narK*, *narG*, and *fnr* under this cultivation condition. The transcription levels of *narK* and *narG* were significantly enhanced in WX-02△AbrB, consistent with the results obtained in original M9 medium (Fig. 4B). Meanwhile, the nitrate utilization capabilities were reported to be significantly improved in NarK and NarG overexpression strains (Supplementary Fig. 4) [43], indicated that nitrate respiration was negatively regulated by transcription factor AbrB, which inhibits the expression of *narK* and *narG*. Notably, the transcription changes of gene *fnr* were completely opposite before and after the addition of nitrate, which may be related to nitrate regulation [44, 45].

To verify the in vitro interaction between AbrB with genes *narK*, *narG* and *fnr*, we conducted a rational analysis of promoter regions of these genes and prepared the corresponding probes: Bio-PnarK, Bio-PnarG and Bio-Pfnr. The EMSAs demonstrated that addition of 40 μM AbrB protein results in shifts for all probes, with the most pronounced shift observed between AbrB and Bio-Pfnr (Fig. 4C-E). Collectively, our results suggested that AbrB could directly bind to genes *narK*, *narG*, and *fnr* during nitrate respiration.

Furthermore, several studies have indicated that regulatory factor Fnr activates the expression of *narK* and

narGHJI [46–48], which may appear contradictory to our finding that AbrB negatively regulates *narK*. However, considering the spatial and temporal differences between these two regulatory effects, as well as the potential involvement of other intermediate transcription factors, such as ResD, these discrepancies can be reconciled. Under anaerobic conditions, ResD has been shown to initiate the transcription of the *fnr* gene and positively regulate its expression, and Fnr can enhance *narK* gene expression [49]. Besides, as previously mentioned, the indirect regulation mediated by intermediate transcription factors may not manifest during the early stages of cell growth, therefore, we speculated that the indirect regulation of nitrate respiration by AbrB through Fnr might become more evident at later stages. Based on these findings, we proposed an initial transcriptional regulation model of AbrB on nitrate respiratory (Fig. 4F).

Comprehensive regulation model of energy metabolism by AbrB in *Bacillus*

To assess the contribution of AbrB to virous modules of energy metabolism, we conducted a comparative analysis of gene transcriptional changes related to substrate-level phosphorylation, oxidative phosphorylation, and nitrate respiration following the deletion of *abrB*. Using the -△△Ct values of target genes from WX-02△AbrB strain as the ordinate, a bar chart illustrating the effects of *abrB* deletion on these three metabolic modules was generated (Fig. 5A). Comprehensive results revealed a general down-regulation of gene expression related to oxidative phosphorylation in *abrB* deletion strain, while a less pronounced down-regulation trend was observed for substrate level phosphorylation genes, particularly after accounting for low transcriptional levels of homologous genes (*ythA*, *ndhF*, *gapB*, and *acoA*). Furthermore, genes associated with nitrate respiration were significantly up-regulated following *abrB* deletion.

Further investigation of intermediate transcription factors revealed that transcription factor ResD, anaerobic regulator Fnr, and carbon metabolism transcription factors CcpN and CcpA are all directly regulated by AbrB (Fig. 5B). Besides, several studies have reported that these transcription factors can regulate multiple modules. Taking all of this into account, a global model of energy metabolism regulated by transcription factor AbrB in *B. licheniformis* was presented in Fig. 5C. The regulatory model of AbrB appears to follow a pathway-dependent pattern as evidenced by its correlation with target pathways. In the early growth phase, AbrB positively regulated genes involved in glycolysis (*gapA*, *pyk*, *pgk* and *pdhA*), TCA cycle (*mdh* and *sdhC*), substrate level phosphorylation (*pyk* and *pgk*), and oxidative phosphorylation (*sdhC*, *ndh*, *ctaA*, *qoxA*, *cydA* and *atpB*). In contrast, it negatively regulated genes associated with acetoin utilization (*acoA*), branched-chain amino acid catabolism (*lpdV*), gluconeogenesis (*gapB*), and nitrate respiration (*narG* and *narK*). Among them, enhancing the expression of *qoxA*, *sdhC*, *gapB* and *zwf* genes and deleting *cydB* gene have been proved to significantly affect the intracellular ATP supply and increase the intracellular ATP content of engineered strains. The deletion of *abrB* leaded to a significant increase in intracellular acetoin content, providing evidence for its negative regulatory role on acetoin utilization (Supplementary Fig. 5). This observation further underscored the pathway-dependent nature of AbrB's regulatory pattern.

Validation of the universality of AbrB's role on energy metabolism regulation in *Bacillus*

The common motifs of target genes directly regulated by AbrB were analyzed in *B. licheniformis* WX-02, revealing three predicted AbrB-binding sequences: AAAAGCKG (MEME-1), CCGCAAAAHRG (MEME-2), and GCYKKCMTCRNAGCGGSYTKT (MEME-3) (K represents G or T; H represents A, T, or C; R represents A, or G; Y represents C, or T; M represents A, or C; N represents A, T, C, or G; S represents C, or G) (Table 1). However, no obvious regularity in the distribution of these binding sequences within gene clusters (Supplementary Fig. 6). To further investigate these regulatory regions in other *Bacillus* species, putative target genes were screened based on their gene annotations in KEGG database and analyzed using FIMO (Find Individual Motif Occurences) tools. Our results indicated that the aforementioned AbrB boxes were present in genes *pgk*, *pyk*, *ccpN*, *atpI*, *ndh*, *mdh*, *gapA*, and *narG* of *B. subtilis* 168 (GenBank: AL009126.3) (Supplementary Table 5), as well as in the genes RBAM_RS15830, *pyk*, *ccpN*, *atpI*, *hmpA*, RBAM_RS06265, *qoxA*, *sdhC*, RBAM_RS03250, *resD*, RBAM_RS17420, *mdh*, *gap*, *gndA*, RBAM_RS17405, RBAM_RS13185, and RBAM_RS13595 of *Bacillus velezensis* FZB42 (NCBI Reference Sequence: NC_009725.2) (Supplementary Table 6). These findings suggested a lack of conservation in AbrB binding sequence among *Bacillus* species, indicated that DNA sequences bound by AbrB may be more extensive than previously understood.

Discussions

The ever-changing environment often leads to insufficient energy or material supply [4]. It has been reported that bacteria can inhibit physiological processes, such as glucose uptake, under nutrient-restricted conditions, and regulate material and energy metabolism through the mediation of extracellular quorum sensing signaling molecules [3]. Furthermore, at this critical point of growth transition, the adjustment of regulatory states is essential for directing metabolic flow changes [9]. In *B. subtilis*,

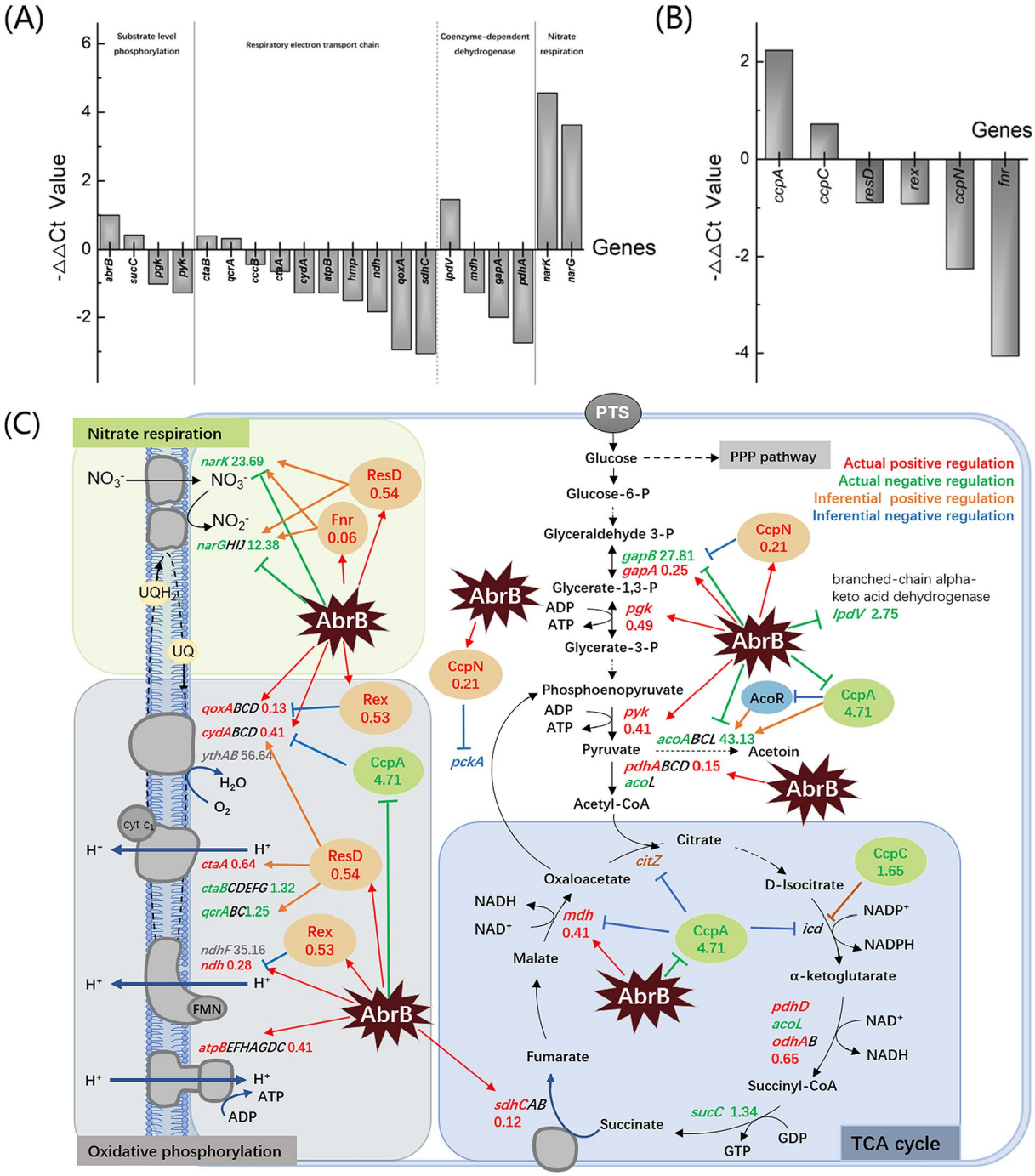
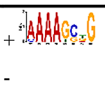
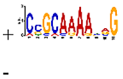
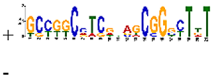


Fig. 5 Comprehensive regulation model of AbrB on energy metabolism in *Bacillus*. **A** Contribution graph of energy metabolism modules. **B** Contribution of intermediate transcription factors. **C** Comprehensive model of energy metabolism

four regulators AbrB, Hpr, Sin and Pai, are expressed during the vegetative growth phase, and can silence gene expression in the later phase [50]. Specifically, the inhibitory effect of Sin is necessary for expression of extracellular protease [51]. Additionally, AbrB is also crucial for the production of alkaline protease AprE in *B. licheniformis*, notably, deletion of *abrB* significantly reduces *aprE* expression, while the removal of AbrB binding site increases transcription level of *aprE* gene by 1.77 times [52]. This highlights how the regulation of transition state can influence global intracellular metabolic changes. By analyzing the regulatory mode of global transcription factor AbrB during the transition phase, this study enhanced our understanding of energy metabolism in bacteria growth and fermentation processes and provided the guidance for engineering transformations.

Table 1 The predicted AbrB-binding sequences in *B. licheniformis*

	Logo	Name	Alt. Name [1]	Width	Motif similarity matrix		
					1.	2.	3.
1.		AAAAGCKG	MEME-1	8	--	0.52	0.36
2.		CCGCAAAAHRG	MEME-2	11	0.52	--	0.35
3.		GCYKKCMTCRNAGCGGSYTKT	MEME-3	21	0.36	0.35	--

[1] "Alt. Name" means "alternate name"

The transition state transcription factor AbrB has been extensively studied in *B. subtilis* [16]. Upon binding to phosphorylated Spo0A, *abrB* is inhibited, leading to the subsequent release of inhibition effects on its downstream genes [11, 12]. From this perspective, AbrB plays a more pivotal role in guiding the transition state. Following stimulation by heat, redox changes, and nitrosation, the σ^H factor induced by bacteria can activate Spo0A expression [6], and this cascade regulation involving SigH-Spo0A-AbrB produces extensive regulatory effects under the influence of AbrB, directing processes such as protease expression [17], spore formation [16], lipopeptide synthesis [19] and antibiotic formation [20] during the middle and late stages of growth. Drawing on the extensive existing literature, this study systematically investigated the regulatory mechanism of AbrB on energy metabolism in *B. licheniformis*, and our results found that AbrB positively regulated the transcription factors CcpN, Fnr, Rex, and ResD, while negatively regulated CcpA, thereby indirectly influenced energy metabolism. Furthermore, three energy production modules were differentially regulated by AbrB. Specifically, AbrB positively regulated substrate-level phosphorylation and oxidative phosphorylation during the logarithmic phage, while negatively regulated nitrate respiration (Fig. 5). This study innovatively linked AbrB to energy metabolism, and ultimately proposed a regulatory model for global transcription factor AbrB during the logarithmic phase and stable phase of cell growth (Fig. 6). It is worth noting that intracellular metabolism in the stationary phase may be more complex. In addition to the regulatory role of AbrB mentioned above, there are also various intermediate transcriptional regulations and post-transcriptional modifications that contribute to this complexity.

Energy metabolism is a critical factor in microbial molecular breeding, and various modification strategies, such as targeting respiratory chain, introducing hemoglobin, and enhancing ATP-dependent enzymes and anaerobic regulatory factor Fnr [26], have been employed to produce diverse array of metabolites, including amino acids [53, 54], polyhydric alcohols [55], terpenoids [56], and extracellular polymers [30, 57]. Although certain regulators associated with energy metabolism have been identified, comprehensive regulatory mechanisms remain unclear, and research focused on enhancing energy metabolism from this perspective is limited. Yamamoto et al. have demonstrated that the expression of *abrB* gene, lacking a Spo0A binding site, could effectively mitigated the growth defect of genome-reduced bacterium *B. subtilis* MGB874, resulting in increased glycolytic flux and elevated transcription levels of genes involved in amino acid and nucleotide biosynthesis [58]. Similarly, in our study, the reduction of cascade inhibition by SigH and Spo0A, along with the enhancement of AbrB protein, leaded to the increases of energy supply and cell biomass, as well as shortened generation time. This approach presented an ideal strategy for strain modification to improve metabolite synthesis (Fig. 1).

Current studies on the modification of AbrB have primarily concentrated on alleviating the direct inhibition of AbrB on metabolite synthesis through deleting gene *abrB*, resulted in the increased production of alkaline protease [59], meropenem [60], antibacterial peptides [61], and surfactin [62]. Xie et al. identified that AbrB plays a pivotal role in enhancing alkaline protease expression through the successive elimination of multiple transcription factor genes [59]. However, the optical density value of *abrB* deletion strain exhibited a significant decline, which may hinder the widespread adoption of this strategy [63]. To achieve high-level production of target products through the engineering of transcription factor AbrB, it is essential to ascertain whether the key genes for target product synthesis are directly regulated by AbrB. In the direct regulation model, the cascading regulatory effects of Spo0A and AbrB should be eliminated to facilitate product synthesis, which involves disrupting the binding interactions between AbrB and target genes. In our unpublished work, this strategy has

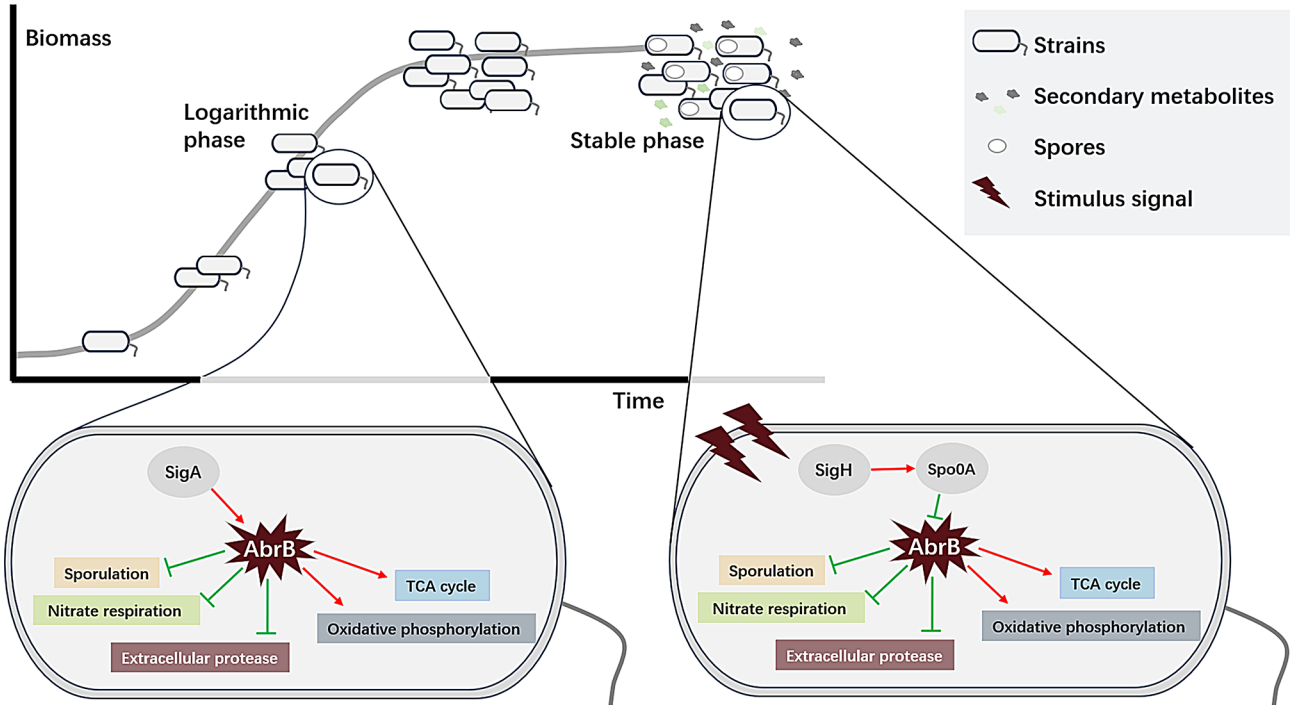


Fig. 6 Regulatory model of AbrB at logarithmic phase and stable phase of cell growth

been shown to increase yields of amino acid polymers. Conversely, in the indirect regulation model, Spo0A and AbrB cascade regulation network should be dismantled. Additionally, the strategies such as promoter replacement or mutations in the protein-DNA binding regions could effectively contribute to achieving these production goals [64].

This study revealed the involvement of AbrB in the regulation of NADPH-dependent dehydrogenases, specifically Icd, GndA, and Zwf, and suggested that AbrB could influence the supply of intracellular coenzyme II and reducing power (Supplementary Fig. 7). Besides, the expression of acetyltransferase gene *yodP* was also regulated by AbrB (Supplementary Fig. 8). Previous acetylome data have demonstrated that energy metabolism related genes *resD* and *atpD*, are regulated by acetylation modifications in *B. subtilis* [65]. Thus, it is suggested that AbrB may regulate energy metabolism through acetylation modifications, further study was warranted to fully elucidate the specific regulatory mechanism.

In summary, this study systematically explored the target genes and transcription factors involved in the energy metabolism of *B. licheniformis*, and established a global network model of AbrB's role in regulating energy metabolism. This research enhanced the understanding of regulatory scope of AbrB, contributing to the comprehension of energy fluctuations during the transition state and laying a theoretical foundation for regulatory network of energy metabolism in *Bacillus*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-024-02572-1.

Supplementary Material 1

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Author contributions
QZ, DBC and SWC designed the research. QZ performed the major experiments and wrote the manuscript draft. QZ, WYZ, SSH, JQL, LSX, SYH, and ZZ analyzed the data and drafted and revised the manuscript. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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