RNA-Binding Protein Hfq: A Role in Cellulose Decomposition

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Original Research Paper

Keywords: *Bacillus subtilis*, cellulose decomposition, cellulase, eglS, Hfq

Posted Date: February 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-157163/v1>

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Abstract

Objective

To study the function of the RNA-binding protein Hfq in *Bacillus subtilis* cellulose decomposition.

Results

In the medium with sodium carboxymethylcellulose (Na-CMC) as the sole carbon source, the knockout of Hfq resulted in a $38.0\% \pm 2.1\%$ and $76.6\% \pm 7.1\%$ decrease in cellulose hydrolysis ability and cellulase activity, respectively. The results of real-time quantitative PCR revealed that several cellulase genes (*eg/S*, *bglA*, and *bglC*) were significantly downregulated in the Hfq knockout strain. The isogenic ΔHfq complemented strain recovered the cellulose hydrolysis ability, cellulase activity, and expression level of cellulase genes. In addition, the survival of Hfq mutant in stationary phase was significantly affected.

Conclusion

RNA-binding protein Hfq is involved in the regulation of cellulose hydrolysis ability, cellulase activity, cellulase gene expression, and stationary phase survival.

Introduction

The development of new eco-friendly bioethanol fuels is crucial to reducing the consumption of fossil fuels, but the bioconversion of cellulose into bioethanol remains a challenge (Zhao et al., 2020). Cellulose cleavage is catalyzed by a group of enzymes referred to as cellulases, including endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91), and β -glucosidase (EC 3.2.1.21) (Garcia et al., 2018; Sadhu and Maiti, 2013). All three enzymes have been used to produce bioethanol; however, the efficiency of cellulase in the existing industrial *Bacillus subtilis* strains remains at a low level (Gu et al., 2018; Zhao et al., 2020). Therefore, it is of great importance to deepen our understanding of the underlying regulatory mechanisms of *B. subtilis* cellulase and to develop new *B. subtilis* strains with more efficient cellulase for industrial applications.

Endoglucanase encoded by the *eg/S* gene is the main cellulase in *B. subtilis*, and can be activated and transcribed by a σ^A -dependent promoter (Robson and Chambliss, 1987). σ^A (encoded by *sigA*), as well as σ^B (encoded by *sigB*), are found in *B. subtilis* and are σ factors, which are components of the multiple-subunit bacterial RNA polymerase (RNAP) and play important roles in response to environmental stresses (Haldenwang, 1995). 6S RNA, an antagonist of σ^A , can compete with a σ^A -dependent promoter in binding with σ^A and then produce the 6S RNA- σ^A -RNAP complex, resulting in transcription inhibition (Trotchaud and Wassarman, 2005).

As the first identified small non-coding RNA (sRNA) in bacteria, 6S RNA can regulate a variety of metabolic processes and other sRNAs (Bobrovskyy and Vanderpool, 2013; Hindley, 1967). Previous

studies revealed that sRNAs often need the incorporation of the RNA-binding protein Hfq to function well (Updegrave et al., 2016; Vogel and Luisi, 2011). 6S RNA encoded by gene *ssrS* has been demonstrated to be a Hfq-associated sRNA in *Escherichia coli*, but the underlying mechanism and functional roles of this interaction remain unclear (Zhang et al., 2003). Considering that the functional and structural properties of Hfq and 6S RNA are highly conserved in most bacteria (Barrick et al., 2005; Sun et al., 2002; Vogel and Luisi, 2011), *B. subtilis* Hfq (Hfq_{BS}) may interact with 6S RNA. As 6S RNA and σ^A are involved in the activation of cellulase genes such as *egfS* (Robson and Chambliss, 1987; Trotochaud and Wassarman, 2005), it is reasonable to hypothesize that Hfq may participate in the regulation of cellulose metabolism via 6S RNA.

To analyze the cellular roles of Hfq_{BS} in more detail, we constructed an Hfq mutant (*B. subtilis*168 Δ Hfq) and its complemented strain (*B. subtilis*168 Δ Hfq-C). We studied the effects of Hfq_{BS} on bacterial growth, cellulose hydrolysis ability, cellulase activity, and cellulase gene expression, which were used as the theoretical basis to determine whether Hfq_{BS} acted as a key factor in cellulose stress adaptation. In addition, the potential relationship between Hfq_{BS}, 6S RNA, *sigA*, and *egfS* was also explored.

Materials And Methods

Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in **Table S1**. *Bacillus subtilis* strains were grown at 37°C in LB medium, Na-CMC medium (70 mmol K₂HPO₄, 5 g Na-CMC, 30 mmol KH₂PO₄, 25 mmol (NH₄)₂SO₄, 0.5 mmol MgSO₄, 0.5 mmol CaCl₂, 10 μ mol MnSO₄, 5 μ mol MnCl₂, and 1000 mL of ddH₂O at pH = 7.2), or on Congo red plates (2 g (NH₄)₂SO₄, 2 g Na-CMC, 1 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.5 g NaCl, 20 g agar, and 1000 mL of ddH₂O at pH natural). *Escherichia coli* strains were cultured at 37°C in LB medium or on a Luria agar (LA) plate (LB with 1.5 % Agar). When required, the antibiotics tetracycline (10 μ g/ml), ampicillin (100 μ g/ml), and erythromycin (200 μ g/mL) were added to the growth media.

Hfq mutant and complemented strain construction

The Hfq mutant was generated from *B. subtilis* 168 by allelic homologous recombination. Briefly, Hfq flanking regions and erythromycin resistance genes (*emr*) were amplified by PCR using the primer pairs UP F/R, DOWN F/R, and EMR F/R (**Table S2**). The upstream, downstream, and *emr* PCR products were digested with SacI/SmaI, XbaI/SbfI, and SmaI/XbaI, respectively. The digested fragments *emr*, down, and up were then ordinal ligated into the clone vector pUC18 to obtain the recombinant plasmid pUC18-*emr*, pUC18-*emr*-down, and pUC18-up-*emr*-down, respectively. The up-*emr*-down fragment of 2556 bp was amplified using UP F and DOWN R as primers. The up-*emr*-down fragment was transformed into *B. subtilis* 168 by Spizizen transformation (Spizizen, 1958). The Hfq mutant, *B. subtilis* Δ Hfq, was obtained after homologous recombination and confirmed by PCR.

To complement the hfq mutant, a DNA fragment from *B. subtilis* 168 containing the entire *Hfq* gene was amplified. The amplified fragment was digested with Sall/SmaI enzyme and cloned into Sall/SmaI-digested pHYP43, which contains the P43 strong promoter and results in the pHYP43+*Hfq* plasmid. The complementing plasmid was transformed and one complement mutant strain, named *B. subtilis*Δ*Hfq*-C, was selected on the LA plate with tetracycline resistance.

Determination of growth curve

Pellets of *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq*-C were grown overnight in LB medium. Then, they were sub-cultured on fresh LB and Na-CMC medium for 60 h independently according to the inoculation amount of initial OD₆₀₀ = 0.1. The OD₆₀₀ values were tested at 0, 2, 4, 6, 8, 10, 12, 24, 36, 48, and 60 h.

Determination of cellulose hydrolysis ability

In order to determine the cellulose hydrolysis ability of *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq*-C, all strains were grown overnight in LB medium and adjusted to an OD₆₀₀ = 0.8. 1 μL of each cell sample was dropped onto separate Congo red plates and cultured for 48 h. After 48 h, the surfaces of the plates were covered with Congo red solution (1mg/ml) and reacted for 1 h. The Congo red solution was removed and NaCl solution (1mol/L) was added and left to react for 30 min, after which it was poured out. At this time, transparent hydrolytic circles of different diameters appeared on the Congo red plate according to the ability of the strain to hydrolyze cellulose.

Determination of cellulase activity

Cellulase activity was determined according to the operational instructions of the kit ([Cellulase \(CL\) test kit](#), Nanjing Jiancheng Bioengineering Institute, China). In short, *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq*-C were grown overnight in LB medium, and then sub-cultured on fresh Na-CMC medium for 60 h independently according to the inoculation amount of initial OD₆₀₀ = 0.1. Cellulase activity was measured at 0, 12, 24, 36, 48, and 60 h.

RT-qPCR

Bacillus subtilis, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq*-C were cultured in Na-CMC medium until the stationary phase (36 h), and total bacterial RNA was extracted using a bacterial total RNA extraction kit (RNAiso Plus, Takara, Japan). A cDNA synthesis was carried out according to the instructions of the reverse transcription kit (PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), Takara, Japan). A two-step RT-qPCR assay with the Bio-Rad CFX96 Real Time PCR System (Bio-Rad, America) was carried out using the SYBR green RT-qPCR kit (TB Green® Premix Ex Taq™ II, Takara, Japan). In order to study the potential relationship between Hfq and cellulose stress adaptation of *B. subtilis*, 11 genes (*eglS*, *bglP*, *bglA*, *bglC*, *licB*, *licC*, *licA*, *ccpA*, *sigA*, *bsrA*, *bsrB*) closely related to cellulose decomposition were chosen for RT-qPCR (**Table S3**). The *16S rRNA* gene was used as an endogenous control (Jagtap et

al., 2016). The relative fold change in target gene expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The growth curve, cellulose hydrolysis ability, cellulase activity, and the expression of cellulase-related genes were evaluated three times in each group to reduce error. Significant differences were determined by an alpha level = 0.05 using one-way analysis of variance (ANOVA) in IBM SPSS statistics software (version 20.0).

Results

Hfq knockout attenuates B. subtilis growth

A mutant strain, *B. subtilis* Δ Hfq, was generated by homologous recombination and confirmed by PCR (**Figure S1**). Wild type *B. subtilis*, mutant *B. subtilis* Δ Hfq and its isogenic complemented strain *B. subtilis* Δ Hfq-C were cultured in Luria-Bertani (LB) and Na-CMC medium separately to examine their growth curve. In LB medium, no significant difference was observed from 0 to 6 h; from 8 h to 60 h, the optical density at 600 nm (OD₆₀₀) of *B. subtilis* Δ Hfq decreased by $10.9\% \pm 3.2\%$ on average compared with that of *B. subtilis* (**Figure 1A**). In contrast, in Na-CMC medium, no significant difference was observed from 0 to 12 h; from 24 h to 60 h, the OD₆₀₀ of *B. subtilis* Δ Hfq decreased by $26.5\% \pm 5.2\%$ on average compared with that of *B. subtilis* (**Figure 1B**). Therefore, our experiments showed that the presence of Hfq gives *B. subtilis* a survival advantage in the stationary phase, whether in LB medium or Na-CMC medium.

Importance of Hfq for cellulose hydrolysis in B. subtilis

All strains were cultured on Congo red plates for 48 h and then stained with Congo red. As shown in **Figure 2** and **Table S3**, the knockout of Hfq reduced the cellulose hydrolysis circle of *B. subtilis* by an average of $38.0\% \pm 2.1\%$, while the isogenic Δ Hfq complemented strain cured the lost phenotype. Therefore, our results showed that Hfq is necessary for *B. subtilis* to maintain efficient cellulose hydrolysis ability, and the existence of the protein contributes to the positive regulation of cellulose hydrolysis.

Positive regulatory effect of Hfq on cellulase activity of B. subtilis

Bacillus subtilis lacks the gene to synthesize exoglucanase, so the cellulase activity measured in this experiment mainly refers to the total enzyme activity of endoglucanase and β -glucosidase (**Accession number: NC_000964**). As shown in **Figure 3**, regardless of whether the observations were noted at 12, 24, 36, 48, or 60 h, the cellulase activity of the Hfq mutant was significantly lower than that of the control group (*B. subtilis*), while the isogenic complemented strain of the Hfq mutant recovered part of the phenotype; the cellulase activity of the Hfq mutant decreased by an average of $76.6\% \pm 7.1\%$. In comparison, the complemented strain of the Hfq mutant recovered an additional $40\% \pm 12\%$ of the

cellulase enzyme activity. These results showed that Hfq has a positive regulatory effect on the cellulase activity of *B. subtilis*.

Hfq regulation of cellulase gene expression in B. subtilis

Tests of cellulose hydrolysis ability and cellulase activity have confirmed that the knockout of Hfq_{BS} seriously hinders the decomposition and utilization of cellulose nutrients such as Na-CMC. To obtain more details of the protein's function in the regulatory network of cellulose catabolism, 11 genes related to cellulose decomposition were used for real-time quantitative PCR (RT-qPCR) analysis (**Table S2**). All 11 genes measured in our study showed significant differences, among which 6 genes (*eglS*, *bglA*, *bglC*, *sigA*, *ccpA* and *bsrB*) were downregulated and 5 genes (*bglP*, *licB*, *licC*, *licA* and *bsrA*) were upregulated in *B. subtilis*ΔHfq (**Figure 4**). The main cellulase gene *eglS* encoded by *B. subtilis* was downregulated by 42.7% ± 10.9% in the Hfq mutant (**Figure 4A**). In addition, after the knockout of Hfq, the β-glucanase genes *bglA* and *bglC* were downregulated by 40.4% ± 7.8% and 63.1% ± 7.7%, respectively (**Figure 4A**). The isogenic ΔHfq complemented strain recovered the lost phenotype. Hence, our results confirmed that Hfq is a core regulatory factor that can positively regulate the expression of cellulase genes in *B. subtilis*.

Discussion

Hfq-mediated B. subtilis survival advantages in the stationary phase

The results of the growth curve are similar to those observed by Hermann and Rochat *et al.*, that is, the existence of Hfq benefits *B. subtilis* survival (**Figure 1**) (Hermann et al., 2014; Rochat et al., 2015). Compared with those in LB medium, the growth defects caused by Hfq knockout are more intense in Na-CMC medium (**Figure 1**). One possible explanation is that Hfq is more important for the survival of *B. subtilis* in a nutrient-poor environment than in a nutrient-rich environment. In addition, the decrease in survival rate after 48 h may be related to the lack of nutrients.

Aid of Hfq in B. subtilis adaptation to cellulose stress

The positive regulation of cellulase activity by Hfq further explains the advantages given by this protein in cellulose hydrolysis ability and stationary phase survival under cellulose stress, since the cellulase activity of *B. subtilis* is closely related to the rate of obtaining nutrients regardless of being in Na-CMC medium or on Congo red plates (**Figure 1-Figure 3** and **Table S3**). High-fiber conditions constitute a stress environment for *B. subtilis* (Ziyao et al., 2015). The possible role of Hfq_{BS} in stress adaptation has been emphasized by this study; nevertheless, it remains to be confirmed.

In *B. subtilis*, the two promoters that initiate Hfq transcription are regulated by σ^B and σ^H (encoded by *sigH*), respectively (Jagtap et al., 2016). σ^H -dependent Hfq transcription results in the synthesis of monocistronic transcripts, while σ^B -dependent transcription results in the synthesis of polycistronic transcripts (*ymaF-miaA-Hfq*) (Jagtap et al., 2016). The monocistronic transcription driven by σ^H may be closely related to the role of Hfq in general life activities such as DNA replication and DNA compaction

because a large number of monocistronic transcripts are also detected in a variety of non-stress environments (Antoine et al., 2018; Dambach et al., 2013; Irnov et al., 2010). Contrarily, in *B. subtilis*, σ^B -dependent genes are generally expressed under stressors, such as antibiotics, temperature, salt, and ethanol (Bingyao and Jörg, 2018). Induction of σ^B -dependent genes is also observed during the stationary phase as bacteria experience stress due to nutrient limitation (Benson and Haldenwang, 1992; Jagtap et al., 2016). In the medium with Na-CMC as the only carbon source, the decomposition ability of cellulose determines the number of available carbon sources obtained by *B. subtilis* for survival. Therefore, our results also proved the positive regulatory role of Hfq in the cellulose stress adaptation of *B. subtilis*. The rapid response of σ^B to stress regulation may contribute to the rapid expression of Hfq under cellulose stress (Haldenwang, 1995; Jagtap et al., 2016).

*A hypothesis: Indirect regulation of *eglS* expression by Hfq through weakening stability of 6S-1 RNA*

The positive regulation of Hfq_{BS} on the expression of the cellulase gene further explains the usefulness of this protein in cellulose stress adaptation. Combined with the results of the growth curve, cellulose hydrolysis ability, cellulase activity, and RT-qPCR, the possible regulatory network of Hfq in cellulose decomposition can be proposed (**Figure 5**). Nevertheless, the accuracy of this view needs to be confirmed by further research.

Endoglucanase (encoded by *eglS*) is the main cellulase secreted by *B. subtilis*, and σ^A activity directly determines the transcription level of *eglS* (Bingyao and Jörg, 2018; Robson and Chambliss, 1987). As expected, both *eglS* and *sigA* were downregulated after the knockout of Hfq (**Figure 4**). 6S RNA is an antagonist of σ^A (Trotochaud and Wassarman, 2005). Some bacterial species can transcribe two or more 6S RNAs, such as the 6S-1 RNA (encoded by *bsrA*) and 6S-2 RNA (encoded by *bsrB*) in *B. subtilis* (Hoch et al., 2015). However, the transcriptional levels of the two 6S RNA-coding genes (*bsrA* and *bsrB*) are diametrically opposed in *B. subtilis* (**Figure 4**). The functional and structural properties of Hfq and 6S RNA are conserved in most bacteria; 6S RNA encoded by the *E. coli* *ssrS* gene is a kind of Hfq-associated sRNA (Barrick et al., 2005; Sun et al., 2002; Zhang et al., 2003). Therefore, some interactions may also occur between Hfq_{BS} and 6S RNA. Previous studies have shown that 6S-1RNA is not expressed in the early exponential phase, and the expression of 6S-1 RNA is four-fold higher than that of 6S-2RNA from the late exponential to stationary phase (Beckmann et al., 2011). Similarly, our results also show that the knockout of Hfq_{BS} mainly leads to an increase in the overall transcriptional level of 6S RNA in the stationary phase (among them, the transcriptional level of 6S-1 RNA is also nearly four-fold higher than that of 6S-2RNA) (**Figure 4**). The negative regulation of Hfq on the stability of 6S RNA may be due to the formation of the Hfq-6S RNA complex, which induces the degradation of 6S RNA by ribonuclease E (RNase E). It is well known that the binding of Hfq to some Hfq-associated sRNA causes the sRNA to be degraded (degradation by RNase E recruited by Hfq-sRNA complex) or protected, while the Hfq itself is unaffected and re-released into the cell (Vogel and Luisi, 2011; Zhang et al., 2003). Compared with 6S-2 RNA, 6S-1 RNA was more likely to be degraded by Hfq. In *B. subtilis* Δ Hfq, the level of 6S-1 RNA was nearly three-fold higher than that of wild strains, while 6S-2 RNA was downregulated by 84.9% $\pm$ 3.1%

(Figure 4). On the other hand, 6S-1 RNA is the ortholog of *E. coli* 6S RNA (Barrick et al., 2005). In addition, 6S-2 RNA may be protected by Hfq during the stationary phase, but the significance of this protection needs to be further studied.

Increase in the compensatory activity of the phosphotransferase system from Hfq knockout

In *B. subtilis*, the combination of the complex formed by *ccpA* (catabolite control protein A) and P-Ser-HPr or P-Ser-Crh with the catabolite responsive elements (Cre) of the target operons can lead to carbon catabolite activation (CCA) or carbon catabolite repression (CCR) (Fujjta, 2009). The *ccpA* transcript levels also requires the assistance of a σ^A -dependent promoter (Bingyao and Jörg, 2018). After the knockout of Hfq, the *ccpA* transcript levels decreased by $45.5 \pm 3.1\%$ **(Figure 4B)**. The downregulation of *ccpA* may be related to the decrease of functional σ^A (Bingyao and Jörg, 2018). When the preferred carbon source is limited (such as Na-CMC medium), CCR usually does not exist (Fujjta, 2009). However, four phosphotransferase system (PTS) related genes (such as *bglP*, *licA*, *licB*, and *licC*) that are inhibited by *ccpA* (only in the presence of CCR) are upregulated **(Figure 4B)** (Fujjta, 2009). In Hfq mutants, the transcript levels of the four genes were more than two-fold higher than those in the wild strains **(Figure 4B)**. The PTS is generally composed of non-specific enzyme I (EI), histidine-containing phosphocarrier protein (HPr), and sugar-specific enzyme II (EII), of which, the latter is mainly responsible for the transmembrane transport and phosphorylation of PTS-sugar (Fujjta, 2009). Therefore, the upregulation of these four PTS-EII genes might partly explains the residual cellulose decomposition and utilization ability after Hfq knockout because secondary metabolites produced by extracellular cellulase decomposition cellulose need to be transported through the PTS to enter the cell (Fujjta, 2009). Another important reason is that the expression of the cellulase gene itself can be directly induced by some small molecular soluble sugars produced by secondary metabolites, although the amount of cellulase produced by this induction effect is relatively small (Aro et al., 2001; Fujjta, 2009).

Despite these findings, the regulatory network related to the downregulation of two β -glucosidase genes (*bglA* and *bglC*) in Hfq mutants is not clear. One possible hypothesis is that the decrease in the transcription level of β -glucosidase may be due to the weakening of CCA mediated by *ccpA*. For example, several enzymes (such as *degS*, *degU*, and *serA*) involved in carbohydrate metabolism have been confirmed to be positively regulated by CCA (Bingyao and Jörg, 2018). Identification of Cre sequences of *bglA* and *bglC* will help to further understand the regulatory network of these two genes.

The positive regulatory effect of Hfq_{BS} on cellulose hydrolysis ability, cellulase activity, cellulase gene expression, and stationary phase survival was confirmed. Therefore, Hfq gives *B. subtilis* a survival advantage under cellulose stress. Additional targets and functional identification could further consolidate the important position of Hfq as a core regulator of multiple metabolic pathways in bacteria, and contribute to a better understanding of the overall function of the protein in bacteria.

Declarations

Acknowledgments

This study was financed by Chengdu Giant Panda Breeding Research Foundation (CPF2015-09, CPF2017-12) (Chengdu, China). The material is original research and has not been previously published nor submitted for publication elsewhere. The authors declare no conflict of interest.

Funding: This study was financed by Chengdu Giant Panda Breeding Research Foundation (CPF2015-09, CPF2017-12) (Chengdu, China).

Conflicts of interest/Competing interests: There is no conflict of interest in this study or in the author.

Ethics approval: This study does not involve any experiments that require ethical or moral approval.

Consent to participate: All the authors voluntarily and agree to participate in this study.

Consent for publication: All the authors have read the manuscript and agreed to publish it.

Availability of data and material: All the data involved in this study are uploaded in the manuscript submission system as charts or attachments in the form of electronic documents, so they are transparent.

Code availability No code is involved in this study

Authors' contributions: All the authors have contributed authors, and they have participated in this study in different forms and made important contributions.

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Figures

Figure 1

Growth curves of *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq-C*. ANOVA results are marked near the OD600 value corresponding to each time point. A and B indicates significant difference from control group *B. subtilis* of 0.01 < P < 0.05 and P < 0.01, respectively; C and D indicates significant difference from control group *B. subtilis*Δ*Hfq* of 0.01 < P < 0.05 and P < 0.01, respectively.

Figure 2

Cellulose hydrolysis ability of *B. subtilis*, *B. subtilis* Δ Hfq, and *B. subtilis* Δ Hfq-C. For statistical analysis, see Table S3.

Figure 3

Cellulase activity of *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq-C*. ANOVA results are marked above the error bars. ** indicates significant difference from control group *B. subtilis* of $P < 0.01$; ^^ indicates significant difference from control group *B. subtilis*Δ*Hfq* of $P < 0.01$.

Figure 4

RT-qPCR analysis of cellulase genes of *B. subtilis*, *B. subtilis*Δ*Hfq*, and *B. subtilis*Δ*Hfq-C*. ANOVA results are marked above the error bars. ** indicates significant difference from control group *B. subtilis* of $P < 0.01$; ^^ indicates significant difference from control group *B. subtilis*Δ*Hfq* of $P < 0.01$.

Figure 5

Diagram illustration of sponge-like HfqBS regulating eglS transcription. (A) σ A-RNAP-6S-1 RNA complex resulting in eglS transcriptional inhibition. Solid lines represent proven interactions; Red solid lines represent transcriptional inhibition. (B) Possible interaction between HfqBS and 6S-1 RNA. Solid lines represent proven interactions; Dotted lines represent possible interactions; Green solid lines represent transcriptional initiation.

Supplementary Files

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- [Additionalfiles.docx](#)