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Fusarium culmorum affects expression of biofilm formation key genes in *Bacillus subtilis*

Maryam Khezri[a,b,c], *Gholamreza Salehi Jouzani*[a,*], *Masoud Ahmadzadeh*[b]

[a] *Microbial Biotechnology and Biosafety Department, Agriculture Biotechnology Research Institute of Iran (ABRII), AREEO, 3135933151, Karaj, Iran*

[b] *Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj 31587-11167, Iran*

[c] *Department of Plant Protection, Faculty of Agriculture, Urmia University, Urmia, Iran*

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ABSTRACT

It is known that there is correlation between biofilm formation and antagonistic activities of *Bacillus subtilis* strains; but, the mechanism of this correlation is not clear. So, the effect of the plant pathogen (*Fusarium culmorum*) on the biofilm formation in a *B. subtilis* strain with high antagonistic and biofilm formation activities was studied. The expression of *sinR* and *tasA* genes involved in the biofilm formation was studied in both single culture of bacterium (B) and co-culture with *F. culmorum* (FB) using real-time PCR. The results revealed that the expression of the *sinR* gene in both B and FB conditions was continuously decreased during the biofilm formation period and, after 24 h (B4 and FB4), it reached 1% and 0.3% at the planktonic phase (B1), respectively, whereas the expression of the *tasA* was continuously increased and was 5.27 and 30 times more than that at the planktonic phase (B1) after 24 h, respectively. So, the expression reduction rate for *sinR* (3 times) and the expression increasing rate for *tasA* (6 times) were significantly higher in FB conditions than the B ones. The relative expression of *sinR* in FB1 (planktonic phase), FB2 (8 h), FB3(12 h), and FB4 (24 h) times was 0.65, 0.44, 0.35, and 0.29, whereas the *tasA* gene expression was 2.98, 3.44, 4.37, and 5.63-fold of the one at coordinate time points in B conditions, respectively. The significant expression reduction of *sinR* and increase of *tasA* confirmed that the presence of pathogen could stimulate biofilm formation in the antagonistic bacterium.

Introduction

Bacillus subtilis is known as one of the most important antagonistic (biocontrol agent) and plant-growth promoting bacteria (PGPR) that is isolated from rhizosphere of different kinds of plants.[1–4] *B. subtilis* strains have the potential to produce more than two dozens of different antimicrobial compounds and antibiotics with an amazing variety of structures[5] and also are able to form multicellular structures or biofilm.[6–8] Biofilm formation occurs in many bacterial species in response to diverse environmental conditions such as nutrient depletion and

* *Corresponding author.*
E-mail: gsalehi@abrii.ac.ir (G.S. Jouzani).
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drought, and it is mediated by many mechanical, biochemical, and genetic factors.[8–10] Commonly, a mixture of polymeric compounds (e.g. extracellular polysaccharides, proteins, and DNA) and an aggregation of different microorganisms can be found in biofilms.[11,12] Ability to form biofilm is associated with numerous benefits for its bacteria. For instance, antibiotics are the most common tools to remove bacteria; but, they are not efficient in the biofilm structure.[6,13,14]

Biofilm formation depends on two matrix gene operons, including *yqxM* (*tapA-sipW-tasA* genes) and *epsA-O* (15 genes) which are directly controlled by a repressor SinR and are responsible for the synthesis of amyloid-like fibers and an exopolysaccharide as two major biofilm components.[2,15–19] Derepression is triggered by *sinI* which is activated by phosphorylated Spo0A (Spo0A∼P) as a master and important regulatory protein in biofilm formation process.[2] The *tasA* gene in the operon *yqxM* is the major gene which encodes the protein involved in antimicrobial activities, spore coat assembly, and germination. It is also found in the stationary phase, sporulating cultures, and the biofilm matrix.[16,17,20–22]

The environmental conditions and presence of other organisms like plant pathogens, symbionts, commensalism organisms, and plant hosts can affect biofilm formation; therefore, different biofilm structures such as plaques, slimes, pellicles, and colonies are seen under various conditions.[1,18,21,23,24] Previously, some researchers have shown that there is positive correlation between biofilm formation as well as PGPR and antagonistic activities of *B. subtilis* strains.[1,2,25,26] Bais et al.[1] demonstrated that a *B. subtilis* strain (ATCC 6051) was able to form biofilm-like structures on the roots of *Arabidopsis* plants and protect Arabidopsis from infections by *Pseudomonas syringae*. Chen et al.[2] showed that plant protection by antagonistic *B. subtilis* strains against *Ralstonia solanacearum* depended on widely conserved genes required for biofilm formation, including regulatory genes and genes for matrix production; so, they provided evidence suggesting that matrix production is critical for bacterial colonization on plant root surfaces.

Previously, we isolated and selected some native *B. subtilis* strains which had high biofilm formation potential and antagonistic capability against *Fusarium culmorum*, the causal agent of foot and root rot on wheat. Finally, the strains with high biofilm production and biocontrol potential were selected. The *B. subtilis* strain Bs12 isolated from sugar beet fields in Kermanshah region (Iran) showed high biofilm formation, volatile production, protease activity, and 79.4% and 83% inhibitory effect against *F. culmorum* at laboratory and greenhouse levels, respectively.[27] It was shown that volatile and protease production as well as biofilm formation by this strain and also other selected strains had significantly positive correlation with their antagonistic ability,[27] which coordinated with the previous reports.[1,2] The principal purpose of this investigation was to find a part of the mechanism for correlation between biofilm formation and antagonistic effect at molecular level; therefore, the effects of a plant pathogenic fungus (*F. culmorum*) on forming biofilm in *B. subtilis* (Bs12) were evaluated. To do so, expression of the *tasA* and *sinR* genes in the strain Bs12 was investigated using real-time PCR method both in the presence and absence of *F. culmorum*.

Materials and methods

Microorganisms and culture conditions

The native *B. subtilis* strain Bs12 (GenBank accession number HQ234328) with high potential in biofilm formation and antagonistic activity against *F. culmorum* was used.[27] The bacterial strain was routinely grown on nutrient agar (NA) or Luria-Bertani broth (LB) at 37 °C. For long maintenance, sterile 40% glycerol was used according to Weller and Cooks[28] and, then, transferred to −20 °C. The *F. culmorum* strain was kindly provided by Plant Protection Research Institute of Iran (PPRI), cultivated on potato dextrose agar (PDA) at 27 °C for routine experiments, and transferred to 4 °C for long time maintenance.

Primers designing

Two specific primers pairs, TasA-F (CAA GCC GTT CCA CTG TGT AG)/TasA-R (AAC CGC TCC TGA ATA TGA TGG) and SinR-F (AAA GGC TAC TCA CTA TCA GAA C)/SinR-R (TCT AAT TGA CCA TCG TAT TCG G), were designed using Oligo (National Bioscience Inc., version 5) software for conducting the real-time PCR experiments. These primers amplified 181 bp and 188 bp DNA fragments of *tasA* and *sinR* of *B. subtilis*, respectively. The primer pairs, 16SrRNA-F (GTA ACC TGC CTG TAA GAC TGG)/16SrRNA-R (CTG TAA GTG GTA GCC GAA GC), with the PCR product length of 110 bp were used as the internal control. Primers were designed in order to have the length of about 20–22 bases, G/C content between 40.9% and 55%, and Tm of about 56–59 °C. Length of the PCR secondary structures and dimer formation was controlled using Oligo Analyzer 1.0.3 software. The primers were synthesized by MWG (Ebersberg, Germany).

To evaluate the specificity of the primers, a PCR was performed using genomic DNA of *B. subtilis* (Bs12) and *F. culmorum*. Genomic DNA of the bacterium and fungus was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma–Aldrich, Zwijndrecht, NL) and Core-one™ kit (CoreBio, USA), respectively.

Co-culture of B. subtilis and F. culmorum

Bs12 cells were grown in biofilm growth medium (BGM) containing an LB-based medium plus 0.15 M ammonium sulphate, 100 mM potassium phosphate, pH 7, 34 mM sodium citrate, 1 mM $MgSO_4$, and 0.1% glucose, as described by Hamon and Lazazzera.[29] Sampling was performed under bacterial planktonic and biofilm formation conditions, according to Stanley et al.[11] To obtain planktonic cells, the bacterial cells were grown overnight in BGM medium at 37 °C with shaking at 200 rpm. Afterwards, the medium containing bacteria was divided into two parts, one co-cultured with suspension containing 10^6 spores per mL of *F. culmorum* and another without any fungal treatment (as negative control). Both beakers were put in the above growth conditions for three more hours (OD_{600} = 2.5 for control). At this time, the bacterial medium was diluted with an OD_{600} = 0.1 in fresh medium and, then, the medium containing bacterium and fungus was diluted with

the same amount of fresh medium. An aliquot of contents of each beaker as planktonic cell population (6 mL) was harvested by centrifugation at 8000 rpm for 10 min for RNA isolation. In the second step, both beakers were incubated at 37 °C without shaking; these conditions were necessary to induce biofilm formation in bacteria. The next samples containing 6 mL taken from each beaker were harvested 8, 12, and 24 h after incubation by centrifugation at 8000 rpm for 10 min. All the taken samples were immediately put at −80 °C until RNA extraction. To normalize the experiments, 100 μL of the media containing microorganisms were cultured on NA and bacterial CFU was counted in each sample after 24 h at 37 °C. Before RNA isolation, normalization was performed by diluting the samples containing more bacterial cells as the final bacterial CFU was the same for all the treatments.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from the harvested samples using Aurum Total RNA Mini Kit (Bio-Rad, USA) according to the manufacturer's instructions. The concentration of RNA was quantified using a spectrophotometer (NanoDrop 1000 spectrophotometer-Thermo Scientific). PCR was performed using RNA (0.6 μg) as the template to ensure the absence of genomic DNA contamination in the RNA samples. The temperature profile for PCR consisted of a first denaturation step of 5 min at 94 °C, followed by 40 cycles of 94 °C/1 min for denaturation, 60 °C/1 min for annealing, and 72 °C/1 min for extension. A final extension was carried out at 72 °C/5 min.

Total RNA was transformed into cDNA using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) following the manufacturer's protocol. The temperature program for cDNA synthesis was 25 °C/5 min for the attachment of primers, 42 °C/45 min for cDNA synthesis, and 85 °C/5 min for enzyme inactivating.

Real-time PCR

Real-time PCR was carried out using iCycleriQ real time PCR (Bio-Rad, USA) using IQ™ SYBR® Green Supermix Kit (Bio-Rad, USA) in 96-well plates. After the dilution of cDNA, 1 μL (20 ng) was added to 24 μL of PCR mixture (12.5 μL of IQ™ SYBR® Green Supermix, 1 μL of each primer at 10 pmol/μL and 9.5 μL of RNase-free water). Specific cDNAs were amplified by real-time PCR using the specific primers. The real-time PCR cycling conditions were designated as follows: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 20 s, and the final extension was carried out at72 °C for 5 min. Fluorescence measurements were recorded during each annealing step. To establish a melting curve and confirm the primers' specificity, an additional step starting from 50 to 95 °C was performed. This step included ninety 10s cycles, in each of which there was temperature increase by 0.5 °C and, at the end of each 10s, the emitted fluorescence was recorded. The efficiencies of amplifications were determined by running a standard curve by the serial dilutions of cDNA. Efficiency can be calculated by the formula: $E = [10^{(1/-s)} - 1] \times 100$, where s is the slope of standard curve. For each measurement, a threshold cycle value (C_T) was determined. C_T is defined as the number of cycles required for the fluorescent signal to pass the threshold (i.e. exceeds the background level). Finally, the expression of genes was calculated by formula $2^{-\Delta\Delta Ct}$.[30] The results were normalized using *B. subtilis*16SrRNA gene as the internal gene. The ultrapure water was used instead of cDNA as a negative control and the gene expression levels were compared with the negative control.

Statistical analysis

Measures were taken for each condition by cDNA synthesized from RNA extracted from three independent cultures and performed in triplicate for each gene. Real-time PCR data analysis was performed using Bio-Rad software based on the threshold cycle (C_T). Analysis of variance, comparison of means, and score of treatment groups were obtained using SAS (version 9.1) and Duncan Multiple test ($p < 0.01$).

Results

Primer specificity and real-time PCR optimization

To evaluate specificity of the designed primers, PCR was carried out using genomic DNA of *B. subtilis* (Bs12) and *F. culmorum*. When bacterial genomic DNA was used as the template, TasA-F/TasA-R and SinR-F/SinR-R primers amplified 181 and 188 bp fragments, respectively. In addition, the PCR product of internal control primer was a 110 bp fragment. No PCR product was observed when the fungal genomic DNA or negative control was used. After sampling and RNA extraction, PCR was performed using the samples of RNA and 16SrRNA-F/16SrRNA-R. No fragment was amplified in the samples. These results confirmed that there was no DNA contamination in the RNA samples. To determine the amplification efficiency, different serial dilutions of cDNA were used for each primer. For instance, five dilutions of cDNA from 1 to 0.0001 were used for 16SrRNA primers and, finally, cycle threshold, Tm, and standard carvers were obtained. According to this experiment, the efficiency of 16SrRNA, TasA, and SinR primers was determined as 92.75%, 99.98%, and 96.78%, respectively.

Evaluating effect of pathogen presence on sinR expression

Relative expression levels of *sinR* gene in the strain were calculated in the absence (B) and presence of the fungus (FB) from three independent cultures in triplicate. The results indicated that the maximum expression of *sinR* was observed when the bacterial cells were in the planktonic phase in the absence of *F. culmorum* (B1) (Fig. 1(a)). By entering the biofilm formation phase, the expression of the gene was critically decreased, which was continued over the time from B1 to B4 (24 h after entering the biofilm formation period). The maximum reduction rate of the gene expression was observed 8 h after starting the biofilm formation compared with planktonic phase (about 80% reductions) and the minimum expression was observed 24 h after starting biofilm formation (B4) which was about 1% of the expression level in planktonic phase (Fig. 1(a)). When the bacterial cells were co-cultured with *F. culmorum*, the *sinR* expression reduction trend was critically increased. Similar to the experiments in which plant pathogen was absent, the

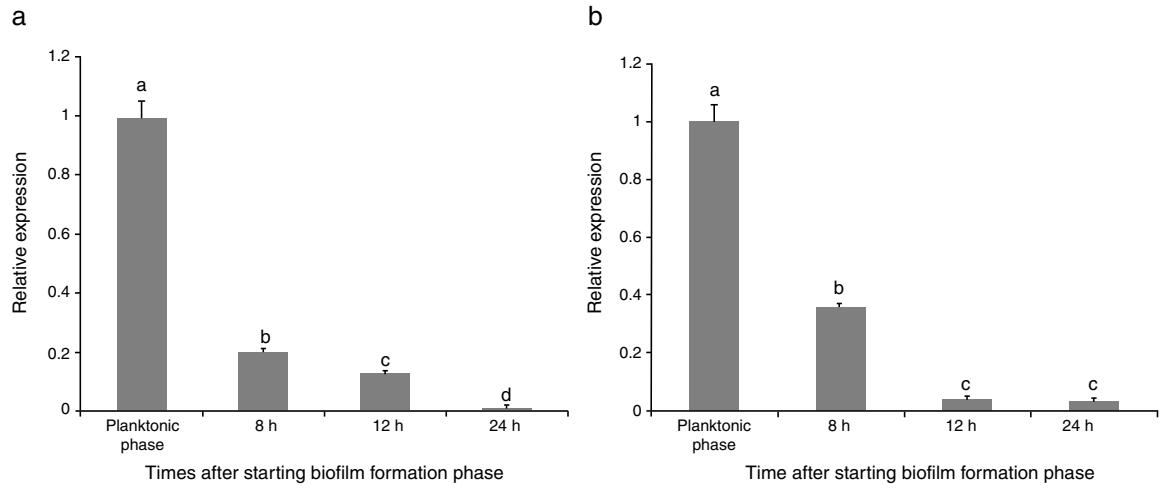

Fig. 1 – Relative expression of the *sinR* gene during biofilm formation period compared with the planktonic phase (a) in the single culture of *B. subtilis* strain (B), (b) in the co-culture of bacteria *B. subtilis* and *F. culmorum* (FB). Different letters indicate significant difference ($p < 0.05$).

maximum expression in the co-culture system occurred when the bacterial cells were in planktonic phase (FB1). It was continuously reduced 8 (FB2), 12 (FB3), and 24 (FB4) h after entering the biofilm formation phase and reached 3% of the expression level in planktonic phase (FB1) (Fig. 1(b)).

The maximum reduction rate of the gene expression was observed 8 h after starting the biofilm formation (about 64% reductions). Comparison of the results of both treatments (B and FB) showed that the relative expression of *sinR* in FB condition was significantly lower than that of B condition in the same growth conditions and time points (Fig. 2(a)). The relative expression for FB1, FB2, FB3, and FB4 was 0.65, 0.44, 0.35, and 0.29 compared with B1, B2, B3, and B4, respectively, and by increasing the time during the biofilm formation period, the reduction rate of the *sinR* gene expression in FB condition was continuously increased compared with that of B condition in the same growth conditions and time points (Fig. 2(a)). The relative expression of the *sinR* gene during the planktonic and biofilm formation period in B and FB conditions compared with the planktonic phase of B condition is shown in Fig. 3(a). The maximum and minimum expressions were observed in the planktonic phase of B condition (100%) and 24 h after starting the biofilm formation in FB treatment (0.3%), respectively (Fig. 3(a)).

Evaluating effect of the pathogen presence on expression of tasA gene

The results of quantitative PCR showed that the expression of *tasA* gene continuously increased from planktonic to the final biofilm formation phases in B condition (B1–B4). The maximum gene expression was observed when the bacterial cells were at 24 h after starting the biofilm formation period in the absence of *F. culmorum* (B4), which was 5.27 times more than that in the planktonic phase (B1) (Fig. 4(a)). By entering the biofilm formation phase, the gene expression was critically increased, which was continued over the time from B1 to B4 phases. The maximum increasing rate of the gene expression

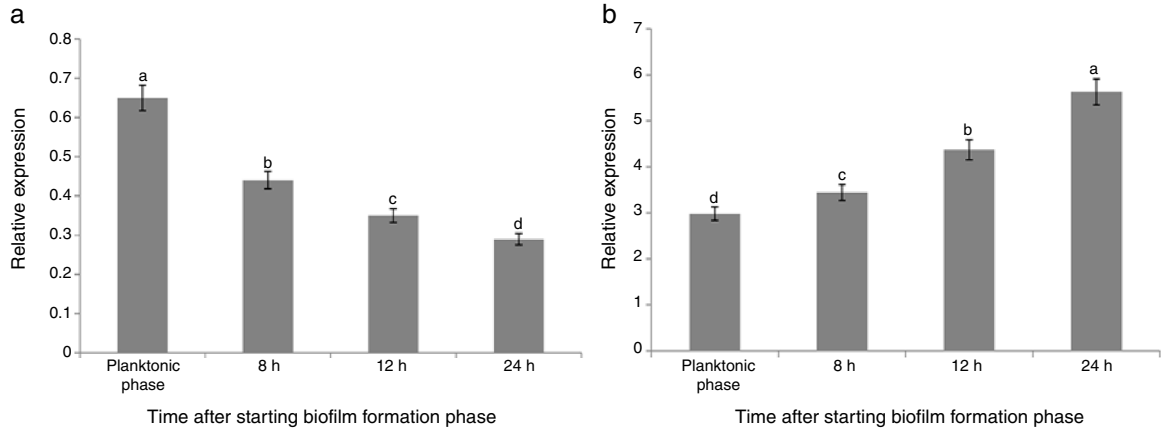

Fig. 2 – Relative expression of the *sinR* (a) and *tasA* (b) genes during planktonic phase and biofilm formation periods in the co-culture of *B. subtilis* and *F. culmorum* (FB) compared with its expression point when the pathogen is absent at each time (comparison of the gene expression of FB_n to B_n). Different letters indicate significant difference ($p < 0.05$).

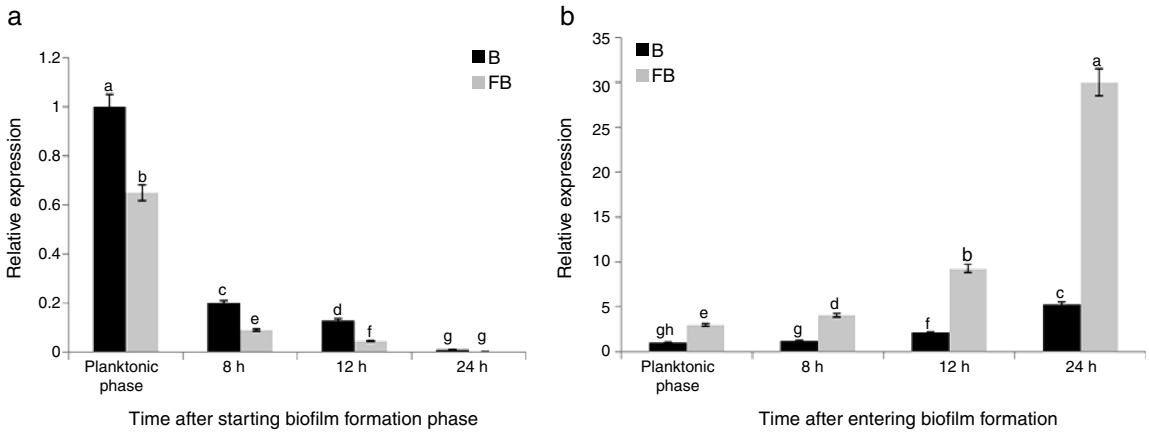

Fig. 3 – Relative expression of the *sinR* (a) and *tasA* (b) genes during biofilm formation period in both single (B) and co-culture (FB) conditions compared with the planktonic phase in the single culture condition. Different letters indicate significant difference ($p < 0.05$).

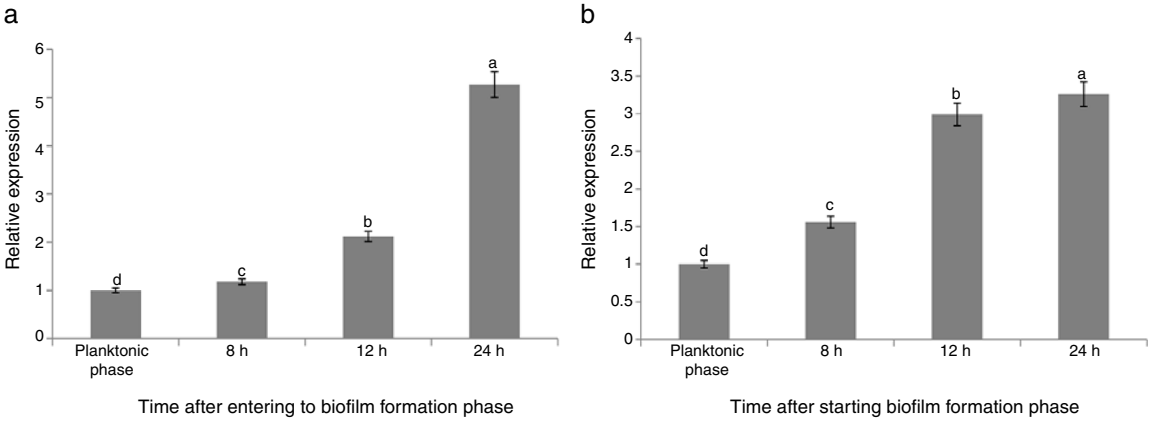

Fig. 4 – Relative expression of the *tasA* gene during biofilm formation period compared with the planktonic phase (a) in the single culture of *B. subtilis* strain (B), and (b) in the co-culture of bacterium *B. subtilis* and *F. culmorum* (FB). Different letters indicate significant difference ($p < 0.05$).

was observed at 24 h after starting the biofilm formation in which the expression of the gene was increased up to 315% compared with the phase B3, whereas the minimum increasing rate was observed at 8 h after starting biofilm formation (B2) in which the expression of the gene was increased by about 18% compared with the previous phase (B1) (Fig. 4(a)).

When the bacterial cells were co-cultured with *F. culmorum*, the *tas*A expression was significantly and continuously increased, which was critically increased during different phases from FB1 to FB4 (Fig. 4(b)). This increasing rate was significantly more than that of the treatments containing only bacterial cells. The levels of expression of the *tas*A gene 8, 12, and 24 h after entering biofilm formation phase were 1.59, 2.99, and 3.26 times more than those of the planktonic phase (FB1) (Fig. 4(b)).

The expression of the *tas*A gene in FB1 to FB4 phases was 2.98, 3.44, 4.37, and 5.63-fold of the one at similar time points in B (B1 to B4) conditions, respectively (Fig. 2(b)). These results suggested that the pathogenic fungus stimulated the expression of *tas*A gene. Fig. 3(b) shows the relative expression of the *tas*A gene during the planktonic and biofilm formation periods in treatments B and FB compared with the planktonic phase of treatment B (B1). The maximum expression was observed 24 h after starting the biofilm formation in treatment FB, which was 30 times more than that of the planktonic phase of treatment B (3000% increase) (Fig. 3(b)).

Discussion

We characterized 30 Iranian native *B. subtilis* strains isolated from the rhizosphere of various hosts in different regions of Iran. The results of laboratory and greenhouse experiments showed that volatile and protease production as well as biofilm formation by some strains had significantly positive correlation with their antagonistic ability and, finally, the most powerful antagonist strains with high biofilm production were selected. Strain Bs12 isolated from sugar beet fields in Kermanshah region showed 79.4% and 83% inhibitory effect against *F. culmorum* at laboratory and greenhouse levels, respectively, and high biofilm formation, volatile production, and protease activity; therefore, it was selected for the present study.[27]

Previously, it has been shown that many different factors such as different fungal compounds (fungal culture supernatant), pH, temperature, nutrient compounds,[31] indole,[13] complex polysaccharides,[32] and oxygen rate affect the biofilm formation. So, to explore the detailed mechanisms of different factors on biofilm formation in *B. subtilis*, it is necessary to perform detailed studies covering all biotic and abiotic environmental factors. Different genetic pathways that are induced by environmental signals are involved in the interaction of cells and abiotic surfaces. These factors can be changed in the amount or type of nutrient content, osmotic factor, pH, temperature, iron, oxidative stress, and substrate type.[33,34] Stanley and Lazazzera[35] showed that environmental signals and regulatory proteins affect the initial steps of bacterial biofilm formation and the nature of mature biofilm structure. So, surface attachment and biofilm formation on different biotic and abiotic substrates are influenced by nature and various environmental stimulations.[24] The presence of other organisms, such as pathogens, is known as one of the factors affecting the biofilm formation and structure in *B. subtilis*; but, the mechanism is not well known.[1,25,26] So, the principal purpose of this investigation was to find a part of the mechanism for correlation between biofilm formation and antagonistic effect at molecular level; therefore, the effects of a plant pathogenic fungus (*F. culmorum*) on forming biofilm in *B. subtilis* (Bs12) were evaluated. To do so, real-time PCR as a sensitive and quantitative technique was used to measure the expression profiles of two important genes (*sin*R and *tas*A) involved in the biofilm formation process of the bacteria in the presence and absence of *F. culmorum*, the causal agent of wheat common root rot. *B. subtilis* is commonly isolated from rhizosphere of different plants, shows antagonistic activities against plant pathogens, and may be used as plant-growth promoting bacteria.[1,3,4] Various microorganisms can be found together in the rhizosphere. The presence and production of metabolites by other microorganisms can be very effective for biofilm formation in target bacteria. Our results indicated that the expression of *sin*R was significantly reduced in the presence of the pathogenic fungus. Expression of this gene was at a high level in the planktonic phase of bacterial growth; but, it decreased upon entering the production of biofilm, as was expected. Several previous studies have demonstrated that *sin*R as one of the most important regulatory genes has a direct negative control on biofilm formation of *B. subtilis*.[17,20,21] Leiman et al.[36] showed that point mutations in the *sin*R gene resulted in a significant increase in biofilm formation *in B. subtilis* and confirmed that it is a key matrix regulatory gene for biofilm formation. Previously, this subject has been also confirmed by other researchers.[37,38] Synthesis of main components of biofilm matrix, such as extracellular polysaccharides and proteins, is mediated by two operons of 15-gene *eps* and three-gene *yqxM*, respectively.[16] Both of these operons are under direct negative control of the *sinR* gene. Indeed, this repressor protein binds to multiple sites within the promoter region for the mentioned operons, thereby repressing its transcription. When this negative regulator is active, expression of these 18 important genes will be suppressed. So, *sinR* gene is known as a master negative regulator in the biofilm formation process of *B. subtilis*.[17] Transcription of the *sinR* gene is controlled by another gene called *sinI*. When bacteria are in biofilm formation conditions, such as environmental stress, shortage of some nutrient sources, etc., transcriptional factor SpoA becomes phosphorylated and activates expression of *sinI*. Activated *sinI* can be prevented from *sinR* expression. Thus, *eps* and *yqxM* operons are activated and, consequently, the genes involved in biofilm formation are expressed.[20] In the present study, it was observed that the expression of *sinR* was decreased in the biofilm compared with the phase of planktonic cells in both treatments. On the other hand, expression of *sinR* in free-swimming cells was higher than that of the sessile ones in the presence or absence of *F. culmorum*. The expression of *sinR* in FB condition was less at each time point compared with B condition. In planktonic cells, the gene expression level was decreased in the co-culture system (FB1) compared with single culture of bacterium (B1). This diminution rate was repeated at each time point of biofilm formation phase, namely FB2, FB3, and FB4 samples compared with B2, B3, and B4 samples, respectively. These results suggested that the presence of fungus in Bs12 growth medium caused a significant reduction of *sinR* gene expression in both planktonic and sessile cells.

In the case of *tasA* gene, opposite results were obtained. In the planktonic phase, expression of the gene was at the lowest level. By entering the biofilm production phase, the expression of the *tasA* gene was increased and, at final time point of biofilm formation (B4), it reached the highest levels of 5.28-fold. Previously, some other researchers have reported these results and shown significant increase in the expression of *tasA* gene during biofilm formation.[20,39,40] This increasing rate was observed in bacterial growth phases in both single and co-culture conditions; but, the increasing rate in FB was significantly more than that of treatments B. Branda et al.[16] showed that TasA is a major protein in biofilm extracellular matrix and the absence of this protein results in a residual matrix. TasA has been detected in stationary phase and sporulating cultures. It appears that TasA has several other functions; for instance, it acts as a broad-spectrum antibacterial factor and seems to have roles in spore coat assembly and germination.[40,41] Evaluation of the relative expression of *tasA* gene showed that the expression in FB treatments was significantly increased compared with B treatments at each time point. Similar results were observed in planktonic and all biofilm formation levels. Based on these data, expression of *tasA* gene increased in the presence of pathogenic fungus. Many transcriptional factors in physiological activities of bacteria are regulated by environmental stress; for instance, biofilm formation by *B. subtilis* stimulated in non-optimal growth conditions. As *F. culmorum* is a pathogenic fungus, its presence in growth medium of the antagonist bacteria has provided a non-optimal condition; consequently, induced and enhanced biofilm formation down-regulate *sinR* and up-regulating *tasA* genes.

In addition to the mentioned key genes involved in biofilm formation in *B. subtilis*, it has been recently demonstrated that other different genes and factors affect biofilm formation. For instance, it has been shown that the genes encoding antimicrobial proteins, such as surfactin and bacillomycin which are involved in the antagonistic activities of bacterium against plant pathogens, significantly and positively affect biofilm formation.[25,26] Gerwig et al.[42] confirmed that the protein

tyrosine kinases EpsB and PtkA differentially affected biofilm formation in *B. subtilis*.

Also, it has been shown that the RapP-PhrP Quorum-sensing system of *B. subtilis* affects biofilm formation through multiple targets due to an atypical signal-insensitive allele of RapP.[43] Recently, complete genome of some biofilm-forming *B. subtilis* strains with antagonistic activities has been sequenced. Also, more detailed information about the correlation of antagonistic activities and biofilm formation is expected to be explored.[44]

In conclusion, according to our results, for the first time, it was shown that the major genes, including *sinR* and *tasA* involved in biofilm formation in *B. subtilis*, were significantly affected by the interaction of bacteria and fungus. Also, the presence of *F. culmorum* stimulated biofilm formation in *B. subtilis*. These findings confirmed that the presence of other organisms, such as plant pathogens in the environment of the bacterium, stimulated biofilm formation. The present study could be the first step to determine the mechanism of relationship between antagonistic activities and a biofilm formation. But, to characterize the detailed mechanisms, it is necessary to perform more detailed studies in this field.

Conflicts of interest

The authors declare no conflicts of interest.

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