Robust Biofilm-Forming Ionizing Radiation Resistant *Bacillus subtilis* isolated from Ramsar-Iran, Emphasis on Molecular Genetic profiling Analysis

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Abstract

Background

The production of structurally complex multicellular communities or biofilms is a mode of growth that is believed to require and involves a differential gene expression as compared to their planktonic counterpart. There is a poor knowledge of biofilm formation and its molecular signaling function in a radiation resistance bacteria. Therefore, the aim of the present study was to investigate the genetic variations and gene expression levels of some critical genes involved in the biofilm formation and matrix production in an ionizing-radiation-resistant *B. subtilis* (RAM-04) isolated from Abe-Siah hot spring, Ramsar- Iran. *Bacillus subtilis* DSM-10 a non-ionizing-radiation-resistant and standard strain model considered as a negative control.

Results

Quantification of biofilms performed using crystal violet assay and Scanning Electron Microscopy that the results presented a significant difference of a high level of the biofilm formation in terms of quantity and structure by *Bacillus subtilis* (RAM-04) as compared to *Bacillus subtilis* DSM-10. At the Molecular level, a total of 13 genes including (tapA) yqxM-sipW-tasA (operon), sinR , sinI , ccpA , epsA-O , spoOB , spoOA , slrA , slrR , ymcA and abrB were selected to study the genetic variations and gene expression levels using direct sequencing method and quantitative PCR technique. Primary analysis revealed genetic variations in (tapA) yqxM-sipW-tasA (operon), sinR , sinI , epsA-O , and slrR in *B. subtilis* (RAM-04) as compared to the negative control. The result of gene expression revealed a significant difference between test and control bacteria. (tapA) yqxM-sipW-tasA operon was significantly over expressed (p = 0.000 with expression folding of 13, 11, 8 respectively) and the expression level of ccpA and slrR were significantly down (p = 0.000 with expression folding of 0.2 and 0.3 respectively).

Conclusions

These results support the hypothesis that the induction of biofilm formation is performed by (tapA) yqxM-sipW-tasA operon signaling pathway, which in turn indicate that this operon has a positive role in biofilm formation in ionizing-radiation-resistant *B. subtilis* (RAM-04) under natural radiation environment.

1 Introduction

Radiation resistance has been detected among bacteria and some microorganisms growing in natural radiation environment. Biofilm formation is one of the specific ability of bacteria response to environmental signals, Lewis,K.,(2001). Biofilms are structured communities of microorganisms that are held together by an extracellular binding matrix typically called exopolysaccharides (EPS), Branda et al., (2005). The model Gram-positive Bacterium Bacillus subtilis has recently been shown to form biofilms in a distinct developmental pathway by different kinds of genes and operon systems that are differentially expressed in a biofilm mode of growth,Branda et al., (2001); Stanley et al., 2003). In B. subtilis,

extracellular matrix contains exopolysaccharides (EPS) and proteins, Branda et al.,(2006).The EPS is produced under the control of epsA-O operon, Branda et al., (2001); Gerwig et al., 2014), and two secreted protein TasA (spore associated protein) and TapA that are encoded within the TapA-sipW-tasA operon provide structural integrity to the matrix, Branda et al., (2006). Both operons are under negative control of the repressor SinR gene as a transcriptional regulator (Kearns and Losick, 2005; Chu et al., 2006). The deletion mutations in any of the genes of TapA-sipW-tasA operon subsequently cause a defective pellicle formation, Vlamakis et al., (2008).In B. subtilis, sporulation as an energy intensive process is a crucial mechanism of vegetative cells that are able to withstand extreme environmental stresses such as radiations, Liaqat, Ahmed, and Jahan, (2013). In this bacterium, about 170 genes are requiring for spore formation (mainly spoOA and its activators such as spoOB,spoOF,spoOH and spoOK) and at least 30 genes are required for timing of spore formation,Meeske et al., (2016). It is believed that the activation of spoOA as a transcription factor and master regulator is a crucial transition stage from planktonic growth to biofilm mode, Yan et al., (2016). Biofilm formation is negatively controlled by the transcription factor AbrB (global regulatory proteins). However, to date, the mechanism of AbrB action is still unclear, Hamon et al., (2004). It has also been reported that the controlling production of EPS matrix is controlled by SinR, as a master regulator that is necessary for biofilm formation and sporulation process, lemon et al., (2008), whereas the increase in the level of SinR blocks the biofilm formation, DeLoughery et al., (2016). The YmdB phosphodiesterase is a recently discovered factor that is involved in the control of SinR activity, Kampf J et al.,(2018). YmdB mutant B.Subtilis exhibit hyperactive SinR and are unable to relieve the repression of the biofilm genes. It is appears that the bistable choice between two mutually exclusive lifestyles including, biofilm formation and motility is primarily governed by the interplay between SinR and YmdB, Kampf J et al.,(2018).SinI is an antagonizer that has repressive effect on sinR, and is indirectly under the control of YmcA protein (Lemon et al., (2007); Chai et al., (2008) Liaqat, Ahmed, and Jahan, (2013). SlrA is another anti repressor that is able to control SinR activity ,Kobayashi, (2008); Chai, Kolter, and Losick, (2009). The action of SinR during the biofilm formation is further complicated by slrR gene which is under the transcriptional control of SinR, and is expressed in the presence of high level of SinI, Chai et al., (2010). In structured multicellular communities produced by B. subtilis, SipW (encoding a signal peptidase that is required for TasA protein sorting from the bacterial cells) and YmcA (with unknown molecular function) are necessary in biofilm formation. It has been reported that mutations in these genes can cause a conspicuous defect in pellicle formation, colony morphology, and blocked biofilm formation in B. subtilis strain 3610, Branda et al., (2004). Mutations in YmcA gene produce a flat colonies and no pellicles structure, Branda et al., (2005). Glucose depletion is another signal transduction that appears to be affecting gene expression pattern during biofilm formation in B. subtilis. Glucose inhibits biofilm formation through the catabolite control protein, CcpA, Stanley et al., (2003). Figure 1 shows a regulatory network of biofilm formation in B. subtilis. The aim of the present study was to investigate the genetic variations and gene expression levels of some critical genes involved in the biofilm formation and matrix production in ionizing-radiation-resistant B. subtilis (RAM-04) isolated from Abe-Siah hot spring, Ramsar- Iran compare with a standard and non-radiation resistant B.Subtilis DSM-10. The Primary results of Scanning electron microscopy and biofilm assay methods expressed a significant robust biofilm formation in ionizing-radiation-resistant B. subtilis (RAM-04) compared to DSM-10.Our

Findings presented different variations in some critical genes involved in biofilm formation in B.Subtilis (RAM-04) and on the basis of these variations, (tapA)yqxM-sipW-tasA operon signaling pathway was significantly over expressed and ccpA and slrR genes were down regulated in ionizing-radiation-resistant B. subtilis (RAM-04).

2 Materials And Methods

2.1 Site selection and sample collection

Novel ionizing-radiation-resistant *B. subtilis* (RAM-04) isolated from Abe-Siah (dark water) hot spring that is situated at Ramsar city in the West of Mazandaran Province-Iran with the highest dose of natural radioactivity in the world (E5039 59.1 N36 53 54.9) Figure 2. This hot spring is a shallow (approximately 50 cm) slow-flowing stream, with a pH of 6.10 to 7.70, and temperatures varying from 28 to 31°C, hence it is classified as a hypothermal spring, Dabbagh et al., (2006). This radioactive spring is well-known to contain radium (^{226}Ra) and thorium (^{232}Th). The equivalent dose around the spring was measured previously to be about 13.48 μSv/h, Dabbagh et al., (2006). *Bacillus subtilis DSM-10* as a non-radiation resistant obtained from agricultural research service culture collection-NRRL (NRRL.NCAUR.USDA.GOV).

2.2 Selective enrichment of ionizing-radiation-resistant bacteria

Water sample were collected using a sterile scoop and were stored at the ambient temperature for future process. 150 ml of Water was suspended in a plastic tube containing 250 ml saline (0.9%) and Tween 80 (0.1%). The serial dilutions (10^{-1} to 10^{-9}) of this suspension were prepared sterile distilled water. About 0.1 ml of each dilution was distributed on nutrient agar (NA) medium containing (peptone 5.0 g, meat extract 3.0 g, agar 15.0 g, distilled water 1000 ml) which were subsequently sealed in plastic bags and incubated at 30 $^{\circ C}$ for 7 to 14 days. Colonies were picked from the plates and sub-cultured for isolation of pure colonies. At first screening stage, the number of c.f.u (colony-forming unit) was determined after 14 days of incubation. All colonies were exposed to Gamma radiation using Gamma cell model 220 ^{60}Co sources at a dose rate of 0.45 KGy min^{-1} at room temperature for radiation resistance at 5-25 KGy in Atomic Energy Organization of Iran and Malaysia Nuclear Agency. The protocol used to evaluate radiation resistance described in previous studies, Ferreira,A.C et al (1997). The *B.Subtilis* (RAM-04) surviving dose of 20 KGy was selected.

2.3 Bacteria detection-16S rDNA analysis

Bacterial cells were cultured overnight in 5 mL Luria-Bertani broth. The cells were then separated from the broth by centrifugation at 12,000 rpm. The bacterial suspension was prepared by suspending the centrifuged/pelleted bacterial cells in 400 μL 1× TE buffer (pH 8.0) which were then extracted for genomic DNA using the DNA extraction GF-1 kit (Vivantis-Malaysia) following the manufacturer's instructions. The purity of extracted DNA was checked by the OD260/OD280.16S- rDNA sequencing performed using universal eubacterial primers; forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-AAGGAGGTGATCCAGCC-3'), Weisburg, (1991). The 16srDNA sequence result was blasted (homology

BLAST) by the NCBI and The Phylogenetic Tree designed using clustal- W method and Neighbor Joining analysis in mega 10 software.

2.4 Biofilm Assay

2.4.1 Solid surface-associated biofilm assay

Solid-surface-associated biofilm formation was estimated by the microtitre plate technique with slight modifications (Morikawa et al., 2006; O'Toole, 2011). The extent of biofilm formation was measured using crystal violet staining. Briefly, the two strains of *Bacillus subtilis* (RAM-04) and standard (DSM-10) were grown overnight in 3 mL Luria-Bertani broth (media was prepared according to manufacturer's instructions), at a temperature of 25°C with aeration (shaker incubator). Following 12 hours incubation, the turbidity of all bacterial cultures were checked at optical density (OD) 600 nm, a good exponential phase growth should be around 0.9-1.0 (10^9 CFU/mL). For biofilm growth, cells were diluted 1:100 into fresh filter sterilized Tryptic Soy Broth (TSB) (media was prepared according to manufacturer's instructions). The medium was supplemented with 1% glucose to stimulate the biofilm formation by *B. subtilis.* A 100 µL of diluted cultures were added to 96-well plastic microtitre plate. The plate was kept standing at 37°C for 24 h without shaking. Four replicates were used for experiment.

2.4.2 Crystal violet assay and biofilm quantification

The surface pellicles and the cultures were carefully removed from the wells by dumping out the medium. Next, each well was gently rinsed twice by submerging it in small tab of phosphate buffer saline, the remaining cells and matrices were stained with 125 µL of crystal violet (0.1 %) solution, and incubated for 15 min at room temperature. The wells were rinsed twice with phosphate buffer saline, and pellicles were photographed.

The CV attached to the biofilm was solubilized in 125 µL of 30% acetic acid in water solution, and incubated for 15 min. 125 µL of solubilized CV was transferred into new flat bottom plates. 30% acetic acid was used as a blank, and the biofilms were quantified by measuring their absorbance at 550 nm wavelength.

2.5 Scanning electron microscopy

Overnight cultures of both bacteria were grown in Luria-Bertani broth at 25°C with aeration. After 12 h of exponential growth, the cultures were checked for turbidity at OD 600 nm (0.9-1.0 =10^9 CFU/mL). Next, 1:100 (TSB+ 1% glucose) diluted cells were transferred to 2 mL round bottom Eppendorf micro-centrifuge tube, and incubated at 37°C for 24 h without shaking. Two replicates were used for each bacterial strain. The tubes were then centrifuged at 8.0 rcf for 3 min to get the biofilm pellets. Supernatant was discarded. All tubes were renumbered, and pellets were primarily fixed by adding 1 mL of 4% glutaraldehyde. All tubes were incubated at 4°C for 6 h. The incubated tubes were centrifuged again at 8.0 rcf for 3 min, and the supernatant was discarded. Three serial steps of washing, centrifugation, and incubation were

applied for all tubes. Next, 1 mL of 0.1 M sodium cacodylate buffer was added to all tubes and incubated for 10 min at 4°C. Next step was the post fixation in which an overnight pellet+buffer suspension was centrifuged at 3.0 rcf for 6 min. Supernatant was discarded, and 1 mL of 1% osmium tetroxide was added. The tubes were vortexed for a few seconds and incubated for 2 h at 4°C. Then, the tubes were washed again with 0.1 M sodium cacodylate buffer for three changes of 10 min each. After fixation with cacodylate buffer, the dehydration was carried out with a series of ascending concentrations of acetone: 35%, 50%, 75%, 95%, and 100% (three changes) for 10 min. In this way, the water was gradually replaced by the organic solvents. Next, the cell suspension was transferred by pipette onto a 1 cm diameter aluminium foil coated with albumin. The biofilms were finally subjected to critical point drying for 30 min, and the specimens were transferred into specimen basket in which they were stacked onto the stub using double sided carbon tape for better resolution. The specimens were then coated with gold in sputter coater. SEM images were taken at the Institute of Bioscience Laboratory using JEOL JSM-IT100 (Japan) Scanning Electron Microscope. This protocol was performed twice to evaluate an accurate performance of biofilm formation for each bacterium.

2.6 Molecular analysis study

2.6.1 Genes mutations detection using PCR assay and direct sequencing

Test and control bacteria were cultured overnight in a Nutrient broth with pH 7.0 and 25°C incubation temperature, with aeration. After 12 h of incubation, the bacterial DNA was isolated in stationary phase of growing using the GF-1 kit (Vivantis-Malaysia). Primers designed using Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/) (Table1) and all PCR reactions were amplified in a 25 µL of total volume including 12.5 µL of master mix (dNTPs, mg^{2+}, Taq DNA polymerase), 1 µL of forward and reverse primers and 9.5 µL of distilled water. Each cycle of PCR consisted of denaturation at 95ºC for 30 s, annealing temperatures at different range based on primers for 45s and a final extension step of 10 min at 72^{oC} was added. The PCR products were checked on 1.5% agarose gel. PCR products were analyzed to detect the probability of gene variations in *B. subtilis* (RAM-04) as compared to control. Direct sequencing was performed on an ABI 3130 Automated Sequencer (XL Genetic Analyzer) using the Big Dye Terminator Version 3.1 Cycle. The sequencing results were analyzed using bio edit and Gene Runner soft wares.

2.6.2 Gene Expression Profiling

2.6.2.1 RNA extraction and cDNA synthesis

RNA extraction was carried out using an RNeasy Mini kit (Qiagen Inc., USA) in stationary phase of bacterial growing. Complementary DNA was synthesized from purified RNA with RT2 First Strand kit (Qiagen Inc., USA).The procedure was performed according to the manufacturer's instructions.

2.6.2.2 Quantitative PCR assay

Gene expression levels of some critical biofilm formation genes in ionizing-radiation-resistant *B. subtilis* (RAM-04) performed using QuantiNOVA^{TM} SYBR Green PCR Kit (Qiagen Inc., USA) and the Eppendorf realplex belong with its analytical software (Qiagen Inc., USA).The primers were designed using online Prime 3 software (http://bioinfo.ut.ee/primer3-0.4.0/). Specificity of primers (Primer Secondary Structures and Amplicon Secondary Structures) was checked using Beacon designer and mFOLD soft wares. Table 3 shows the primer sequences and PCR product sizes for each amplicon used in the present study. A final volume of 20 µL pre-mix was prepared containing: 10 µL of 2× SYBR green PCR master mixes, 0.5 µL of each primer, 0.5 µL of cDNA, and 8.5 µL of RNase-free water. All samples were analyzed in duplicate plus control reactions. The PCR was set to 95°C for 2 min to activate the enzyme, 40 cycles of 5 sec each at 95°C (primary denaturation) followed by 40 sec at 60°C (annealing and synthesis) for each gene of interest. The *16S rRNA* served as internal control (reference gene). To check for possible DNA contamination, a No Template Control (NTC) reaction, containing 2 µL dH_2O instead of cDNA was included for every mRNA in every run. Finally, the dissociation curve was constructed immediately after the PCR run to check and verify the results. The relative quantification values were determined by the ΔΔCT method ($2^{\Delta\Delta Ct}=2^{Ct\ (treated\ cells)\ -\ Ct\ (control\ cells)}$) using REST 2009 software. 2 = the amplification efficiency where the template doubles in each cycle during exponential amplification. All data were presented as mean ± standard deviation (S.D). One-way analysis of variance (ANOVA) with *t*-test was used to test the differences between means. $P \leq 0.05$ was considered significant.

3 Results

3.1 16S rDNA analysis

Species level identification of the Abe-Siah isolate was carried out by 16S rDNA sequencing. The phylogenetic tree performed by clustal W, bootstrap method in neighbor-joining analysis (Unrooted Tree) using mega 10 software. Bootstrap values are given at the branching points. The phylogeny tree showing the position of *B.Subtilis* (RAM-04) within 13 strains (Figure3).

3.2 Solid Surface-Associated Biofilm Assay

B. subtilis (RAM-04) was isolated from water and mud samples obtained from Abe-Siah hot spring in Ramsar, Iran. This isolate was found to survive after exposure to 20 kGy gamma radiation with a viable growth of 20 CFU/ml. The isolate formed highly structured colonies on Nutrient Agar medium that were white, raised and strikingly mucoid in their center. The isolate was also found to form robust pellicles (floating biofilms) and solid surface-associated biofilms in Tryptic Soy Broth (TSB) standing culture (Figure 4-a). The micro titer plate assay allowed for the formation of biofilms on the wall and/or bottom of a 96 micro titer plate and the extent of biofilm formation were measured using the crystal violet (CV) dye. Solubilized CV was quantified in a plate reader at absorbance of 550 nm using 30% acetic acid in water as the blank. The results showed a high level of biofilm formation by *B. subtilis* (RAM-04) as compared to the standard model (Figure 4b,4c). In addition, results analyzed by one-way ANOVA (Minitab 16) revealed that the means of the absorbance taken for the two strains were significantly different.

3.3 Scanning electron microscopy

Scanning Electron Microscopy (SEM) allows for the visualization of surface structures with a three-dimensional appearance at very excellent resolutions. SEM observation of the pellicles produced by *B. subtilis* (RAM-04) showed that the cells were densely packed side by side, and embedded in a matrix. In contrast, *B. subtilis* DSM-10 individual cells were only visible with a sparser matrix (Figure5).

3.4 Polymerase chain reaction assay and direct sequencing

The PCR amplicon and primary results of DNA sequencing revealed the existence of 13 selected genes in ionizing-radiation-resistant *B. subtilis* (RAM-04) (Figure6). In the next step of analysis, direct sequencing of 13 genes revealed multiple kinds of variations in ionizing-radiation-resistant *B. subtilis* (RAM-04) as compared to the control. Out of the 13 genes, seven genes namely *yqxM, sipW, tasA, sinR, sinI, epsA-0,* and *slrR* exhibited the genetic variations as compared to control, and six genes namely *abrB, ymcA, slrA, CCPA, spoOB and spoOA* did not show any genetic variation. All variations were transition or transversion in a single nucleotide, and TA insertion (double nucleotide) were seen in *sipW* gene only. All nucleotides variations with nucleotide position changes are presented in Table 2.

3.5 Quantitative PCR

In order to elucidate the biofilm formation response to ionizing natural radiation as an environmental stress, a genetic expression profiling of nine genes were examined. The results revealed a significant over expression (p=0.000) of three genes including *yqxM, sipW, tasA* (known as *tas* operon) in ionizing-radiation-resistant *B. subtilis* (RAM-04) as compared to the control. The up-regulation folding change was 13, 11 and 8 for *yqxM, sipW, tasA* respectively. It was also remarkable that *slrR* and *ccpA* (without any genetic variation) were significantly down-regulated (p = 0.000) in ionizing-radiation-resistant *B. subtilis* (RAM-04) as compared to the control with folding change of 0.3 and 0.2 respectively. There was no significant correlation (p > 0.05) in *sinI, sinR, epsA* and *spo0B* gene expression profiling. Table 3 shows the q-PCR primers, product size of amplicons and expression level of each gene. The results of expression panel have been presented in Figure 7.

4 Discussion

Ionizing resistant bacteria have extensive diversity, and they are spread in different geographical ecosystems with different ranges of radiation resistance. Natural ionizing-radiation-resistant B. subtilis (RAM-04) isolated from Ramsar hot spring- Iran, was able to form a strong biofilm (as compared to the control) in response to extracellular signals in their natural environments. Primary results of DNA sequencing demonstrated that out of 13 genes, seven genes showed genomic variations as compared to the control. Extracellular matrix of EPS and the protein TasA are the key element in biofilm formation, and their development is regulated by the epsA-O operon, Branda et al., (2006). The (tapA)yqxM-sipW-tasA operon is another key protein that controls the biofilm formation, Chai et al., (2008). Despite the existence of various genomic alterations in the (tapA)yqxM-sipW-tasA operon of B. subtilis (RAM-04) genome,

ample evidence has revealed a high level of gene expression with the folding change of 13, 11 and 8 as compared to the control, which was translated to high level of biofilm formation. This finding was in agreement with the result of biofilm formation assay using microtiter plate assay and Scanning electron microscopy that were previously presented in the results. Although deletion mutations in any of the three-gene operon (tapA)yqxM-sipW-tasA resulted in a defective pellicle formation and defective colony architecture, Branda et al., (2006). Based on our results, these genes did not exhibit any deletion, and the only TA insertion was observed in sipW gene. On the basis of these findings, we propose that genomic variations in this operon might be able to produce a robust biofilm by B. subtilis (RAM-04). In the present study, we also observed that the mRNA levels of CcpA and SlrR were down with the folding change of 0.2 and 0.3, respectively as compared to the control. In B. subtilis, the catabolite control protein, CccpA, is a transcriptional regulator that mediates catabolite repression of plenty of genes in response to glucose and fructose, Cairns, Hobley, and Stanley-Wall, (2014). In addition, through quantitative biofilm assay and DNA microarrays, the catabolite repression has been reported to inhibit the biofilm formation in this bacterium, Stanley et al., (2003). However, this contradicts our findings. An interesting point in our investigation was that despite the down expression of ccpA gene, the biofilm formation was actually induced in B. subtilis (RAM-04). To determine the induction of biofilm formation in B. subtilis (RAM-04), we quantified the level of slrR gene. This gene is under the transcriptional control of SinR, and is expressed in the presence of high level of SinI, Chai et al., (2008). Induction of SlrR stimulates matrix production and biofilm formation (Chu et al., 2008; Kobayashi, 2008; Cairns, Hobley, and Stanley-Wall, 2014). At the molecular level, SlrR, by binding to SinI (SlrR-SinI complex), represses the transcription of motility and autolysis genes with the activation of matrix genes, thereby promoting biofilm formation, Chai et al., (2010). Our findings show a down expression of slrR gene. It seemed that biofilm induction was not associated to SlrR pathway in B. subtilis (RAM-04). On the basis of our findings, biofilm formation in B. subtilis (RAM-04) was found to be associated to the high expression level of (tapA)yqxM-sipW-tasA operon that regulates the production of extracellular matrix to prevent and adapt the gamma natural radiation in their geographical ecosystems.

Declarations

Ethics approval and consent to participate

This research done at Universiti Putra Malaysia under IPS grant, Insentif Putra Siswazah, GP-IPS/2016/9488300 and under contributor's guidance and their cooperation.

Consent for publication

All colleagues read the paper carefully and approved the final manuscript.

Availability of data and materials

All raw data and information such as sequencing results, 16sRDNA analysis and sequence, CT value of gene expression study, melting curve raw data, OD of each sample by nano drop, raw data of radiation

dose from nuclear energy center-Iran and Malaysia are available.

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Competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

DSA and RV conceived of the presented idea, carried out the experiment and wrote the manuscript with support from NMA. YR, SR, MSM and ZS helped supervise the project. NMA supervised the project.

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Tables

Table 1 Gene’s information, over lapping primers sequences and PCR conditions used in this study.

Gene Name	Gene ID	Gene locus	Gene Function /Product	Primers (5' to 3')	Annealing Temperature(°C)	Product size (bp)
yqxM	938532	BSU24640	Hypothetical Protein	F1TCAAGTTACAGTGTTTTACAGGAGGT R1 AATAAGGCGAAAGGTGAGCA	59.65	324
				F2 TTTGCACATATCAGATCAAACG R2 GTCGGTTTTTCATCGCATTT	59.9	345
				F3 GCAAACGGCAGTACATTTGA R3 CGGATGAACGTGTTGAAATC	59.4	320
sipW	938542	BSU24630	Signal Peptidase I Transcription Factor	F1 CGGGGAAGAGGATGAAAAA R1 CAAATGGTCTCCTTGCTTTG	54.5	320
				F2 AGAGCTCCAAAAAGGTGACG R2 AGACATGGTGCTGTCCTTTG	58.8	367
				F3 GCTTTTGTGACGATCAGCAG R3 CAAGCGTACCTGATGCAAAA	59.5	385
tasA	938545	BSU24620	Spore Coat Protein N	F1 ACCTGGCAACAGGTTTCGAT R1 TGGCTGAGGAAATCTTCTGG	60.5	374
				F2 GGATCACTTGCGATCAAAGAA R2 TCCATTTGAACCTGATCGAAG	60.2	365
				F3 TGCAAGCGGTAAAGTCAATG R3 AATACTGGGCCGTCTCTTTTT	59.2	329
sinR	938544	BSU24610	XRE Family Transcriptional Regulator, Biofilm Formation and Sporulation	F AGGAGTTAGTGCCTCTGCTCA R TCACAAGGAAGGTGATGACA	58.5	381
ccpA	935942	BSU29740	Catabolite Control Protein A	F1 TCCAGTAAAAGGAGTGGTTTTAGG R1 ACTTGTTTGCCGAGCATTGT	60	377
				F2 TGAGCAACTCTGACCAAAACA R2 AAACGGAAGGTTCGCTTCTT	60	350
				F3 ACATACAGACATCGCGTTCG R3 GGTGAAGCTTGCTCTTTGTTT	59	499
sinI	938543	BSU24600	SinR Antagonist SinI	F ATACGCTGGCCAATCAATGT R CAAGTATTTTAGGAGGAGAAACTGC	60	249
epsA-0	938582	BSU34370	Hypothetical Protein	F1 GGAAGGCTGAAGGCTATGAA R1 CTGAACGGCAACGTTGATTA	54.7	389
				F2 TGGAAGAAGTTAAGGCGGAAT R2 CTTGATCATCGGGCTTTCC	60	248
				F3GTAGATGAAAGAATGAATGTACAAGG R3TTTTTCTAAAGATCACTCGCTTCA	59	297
spo0B			Sporulation Initiation		59	391

	935956	BSU27930	Phosphotransferase B	F1 AAATGATTGGGAGTGCGAAA R1 GCTTGATCAAACAGATGAAACAG		
				F2 CGGAGAAATTAAGGATTTGTCG R2 TCCTCCGTTCTAAACCGTTAAA	60	318
spo0A	938655	BSU24220	Stage 0 Sporulation Protein A		59.5	348
				F1 TGTAGCAAGGGTGAATCCTG R1 CCTAAATCGACGGCCTTTTT		
				F2 GACGGACTTGCGGTTTTAGA R2 CTCGCGTCGAGATTTTTCTT	59.5	281
				F3 CCATCATCGCAAAGCAGTATT R3 CCAAAACAAAACGCCTCCTA	60	488
slrA	8303165	BSU38229	Transcriptional Regulator SlrA	F GTCTTTTATTGCCCGCATGT R TAAATAGATCCGCCTGTTCG	58	235
slrR	938581	BSU34380	HTH-type Transcriptional Regulator SlrR		60	280
				F1 TTCCCCTATAGATGCGAACG R1 CGGCGGTGAAGAAGAATG		
				F2 GAACAATTCTTCCTTTTCCATCC R2 TGAACGAAATAACGACAGGAA	58	331
ymcA	939581	BSU17020	Hypothetical Protein /Regulator	F1 AGGAGAAGCAATCGAGGTGA R1 CCTGGATAACAGGAATCTCTTCA	59.9	294
				F2 AGGAGAAGCAATCGAGGTGA R2 TTCCTGGATAACAGGAATCTCTTC	60.5	285
abrB	937009	BSU00370	Transition State Regulatory Protein AbrB	F CAAATAAGTATCTCTTGGGAGGAG R CGTGAAGTGTACGATGCTTACC	58.5	400

Table 2 Explanation of nucleotide changes, Nucleotide Positions and protein variations in ionizing-radiation-resistant *Bacillus subtilis* (RAM-04).

Gene Name	Nucleotide Variation/ Nucleotide Position	Transition /Transversion	Protein Variation
yqxM	GTG to GAG(1660658), AAA to AGA(1610251), GGC to AGC(1660756),AGG to ACG(1660910),CCG to CAG(1661002),AAA to AAT(1661055)	Transversion, Transition, Transition, Transversion, Transversion, Transversion	Val to Glu, Lys to Arg, Gly to Ser, Arg to Thr, Pro to Gln, Lys to Asn
sipW	CTT to TTT, TA Insertion(1661738-1661739)	Transition, Insertion	Leu to Phe , TA insertion
tasA	GAA to AAA(1661890), TCT to TTT(1661990), CGG to TGG(1662025), TAG to CAG(1662508)	Transition, Transition, Transition, Transition	Glu to Lys, Ser to Phe, Arg to Trp, Stop to Gln
sinR	CGC to TGC(1662700)	Transition	Arg to Cys
sinI	TAA to CAA(1663108)	Transition	Stop to Gln
epsA-0	GTT to ATC(686070,72), ATG to GTG(586348)	Codon change, Transition	Val to Ile, Met to Val
slrR	TTT to CTT(585360)	Transition	Phe to Leu

Table 3 Primer sequences, q-PCR conditions and expression profiling of genes. *yqxM, sipW, tasA* (operon) were highly expressed. *ccpA* and *slrR* showed a down-regulation of expression panel. *16S rRNA* served as reference genes (internal control).

Gene Name	T/R	Primers (5' to 3')	Annealing Temp(˚C)	Product size (bp)	Expression	STD. Error	95% C.I	*p*.v	Result
yqxM	Target	F TTTCGATCACGTTCCCATCC R TTTCGCCTTATTTGCTGTGC	60	122	13.433	7.012-27.680	5.497-33.513	0.000	UP
sipW	Target	F TGGAGCTCTTTCACATCAGTG R AGTTCTGTCAGGTTCGATGG	60	84	11.232	9.711-13.036	9.254-13.646	0.000	UP
tasA	Target	F CGTACCTGATGCAAAAGTAGC R TCTGCAGCACTAGGATTAGC	60	93	8.384	4.769-16.369	3.540-20.464	0.000	UP
slrR	Target	F TACAAGCCGGGATGGAAAAG R CGGTAAGAGGCAGTTTCAGG	60	82	0.388	0.309-0.491	0.288-0.525	0.000	DOWN
sinR	Target	F CTGGACGTCTCGGTTCATAC R GCATCGCGAACCAATTTCTC	60	95	0.375	0.186-0.818	0.145-0.995	0.497	-
ccpA	Target	F AACGCGATGTCTGTATGTCC R TCCAGTGCCGATTGTACTTG	60	132	0.211	0.163-0.274	0.156-0.286	0.000	DOWN
sinI	Target	F AATGGTGGAAGCCAAAGAGG R TTACGGTATGACTTCTGGCTG	60	110	0.315	0.237-0.418	0.232-0.427	0.167	-
epsA-0	Target	F AAGCCGATTCAGATAGGTGC R AATCTCAGTGACATCCAGCG	60	115	0.375	0.300-0.491	0.249-0.573	0.167	-
spo0B	Target	F AGCTTTGATTCGTGCTTTGC R ATTCCCGGCATGATTGGATG	60	124	0.354	0.202-0.638	0.175-0.723	0.167	-
16S rRNA	Reference	F TGATTTGACGTCATCCCCAC R AGCGCAACCCTTGATCTTAG	60	96	1.00	-	-	-	-

Figures

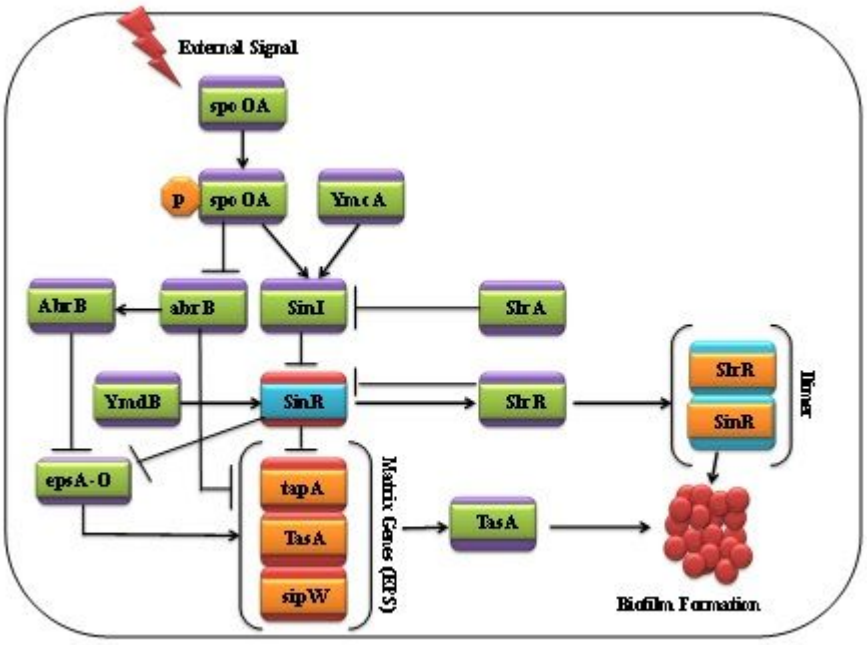

Figure 1

A schematic representation of the complex regulatory pathway (named as Spo0A pathway) that controls the gene transcription during biofilm formation in Bacillus subtilis. Arrows indicate gene activation, and T-bars indicate repression, adapted from Vlamakis et al., (2013) and Kampf, J et al., (2018).

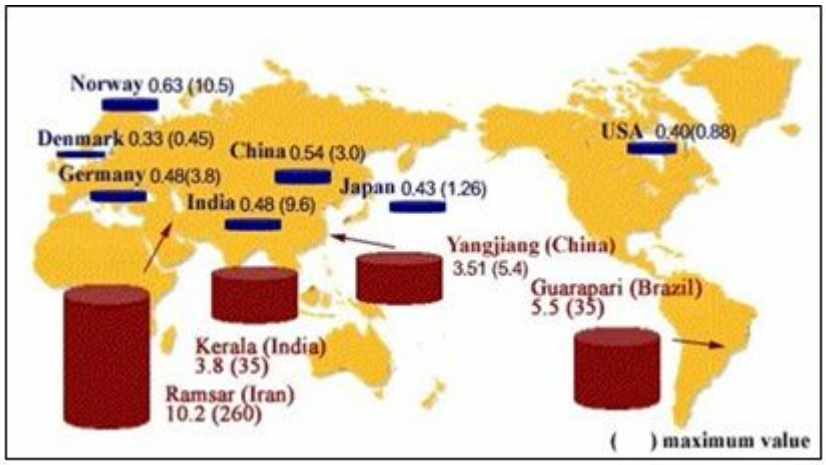

Figure 2

Ramsar location and High natural radiation background of the world, Numbers given are in mSv/year (Ghiassi-nejad et al, 2002, adapted from Sohrabi, 2013).

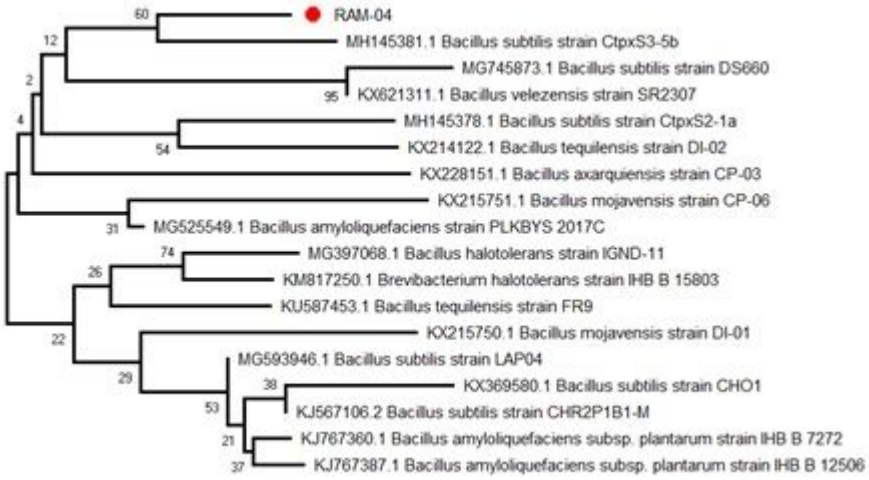

Figure 3

BLAST analysis of B. subtilis- RAM-04 isolated from Abe-siah hot spring-Ramsar. The phylogenetic tree of RAM-04 position within 13 strains that performed by clustal W, bootstrap method in neighbor-joining analysis (Unrooted Tree) using mega 10 software. Bootstrap values are given at the branching points.

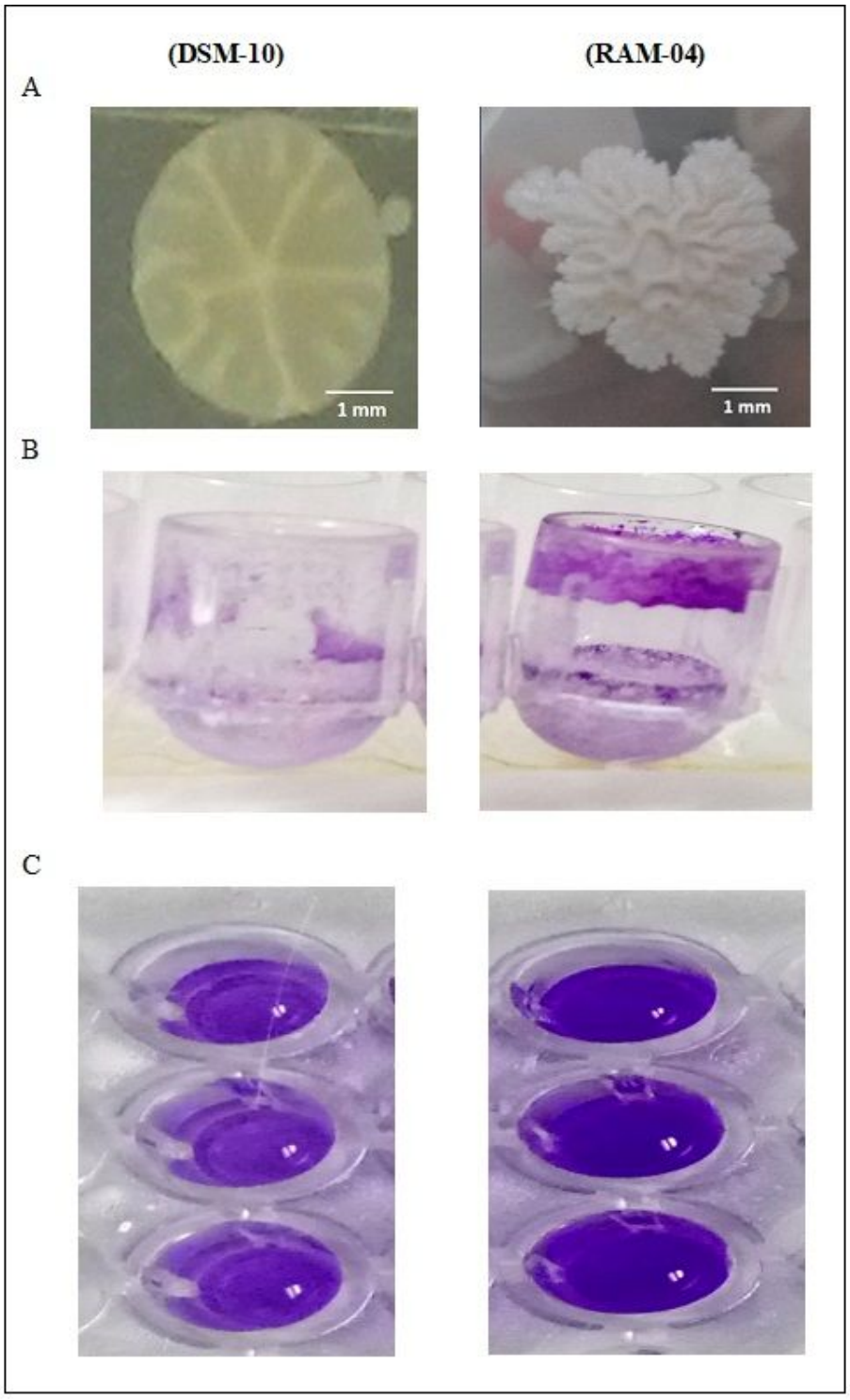

Figure 4

A Comparison of B. subtilis (DSM-10) and (RAM-04) overnight colonies on Nutrient Agar (A), solid surface-associated biofilms with CV staining (B), solubilized CV in 30% acetic acid in water (C).

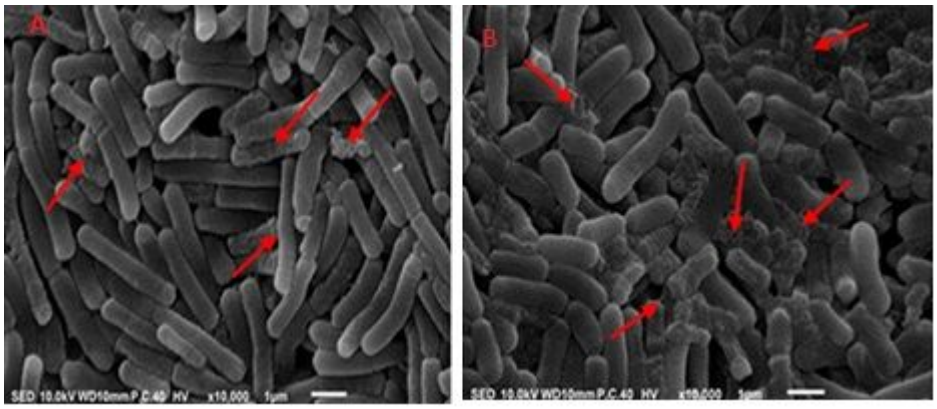

Figure 5

Scanning Electron Microscopy images of pellicles formed by (A) B. subtilis (DSM-10), and (B) B. subtilis (RAM-04). Red arrows indicate the extracellular matrix.

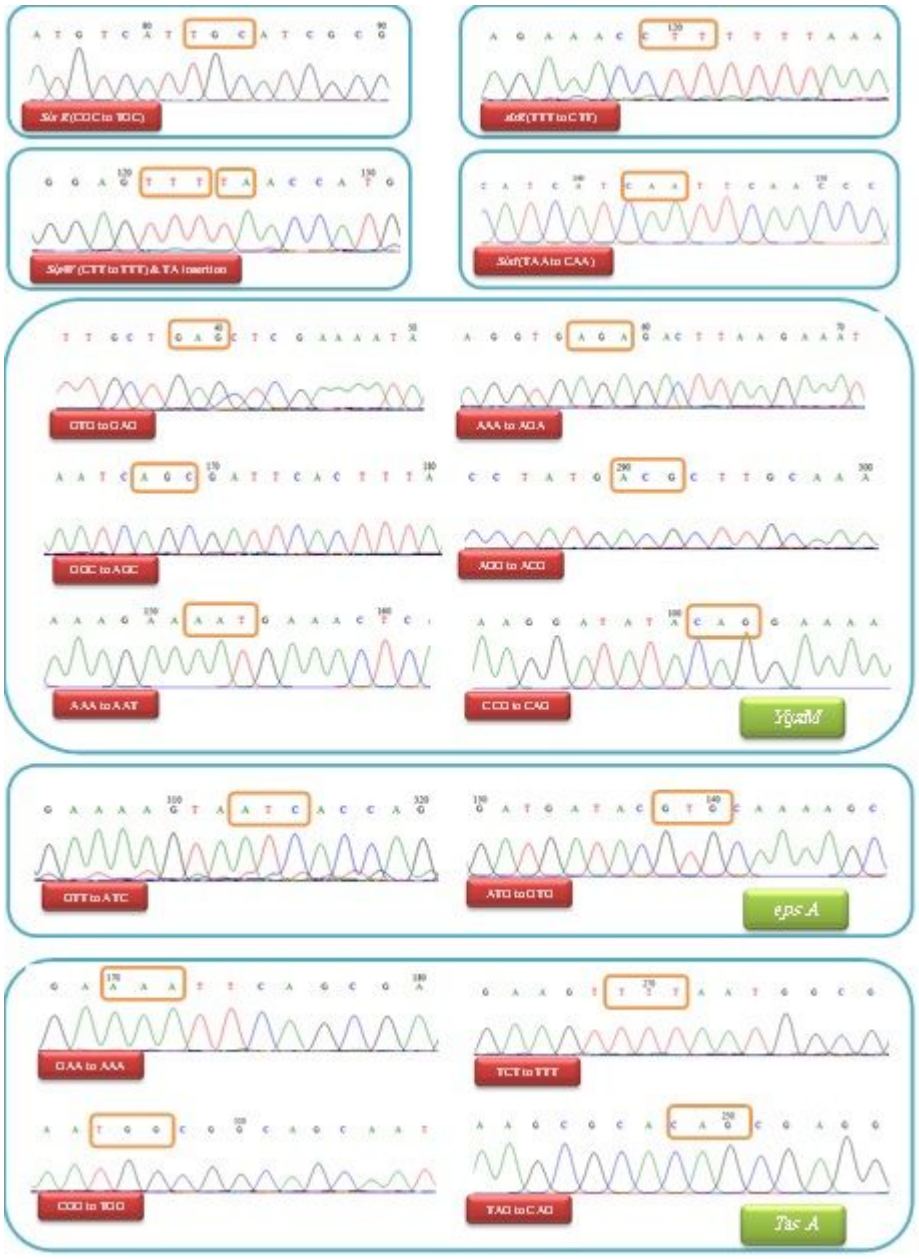

Figure 6

A schematic diagram of sequence chromatograph of seven genes direct DNA sequencing in ionizing-radiation-resistant Bacillus subtilis (RAM-04). Diagram shows genetic variations in each gene.

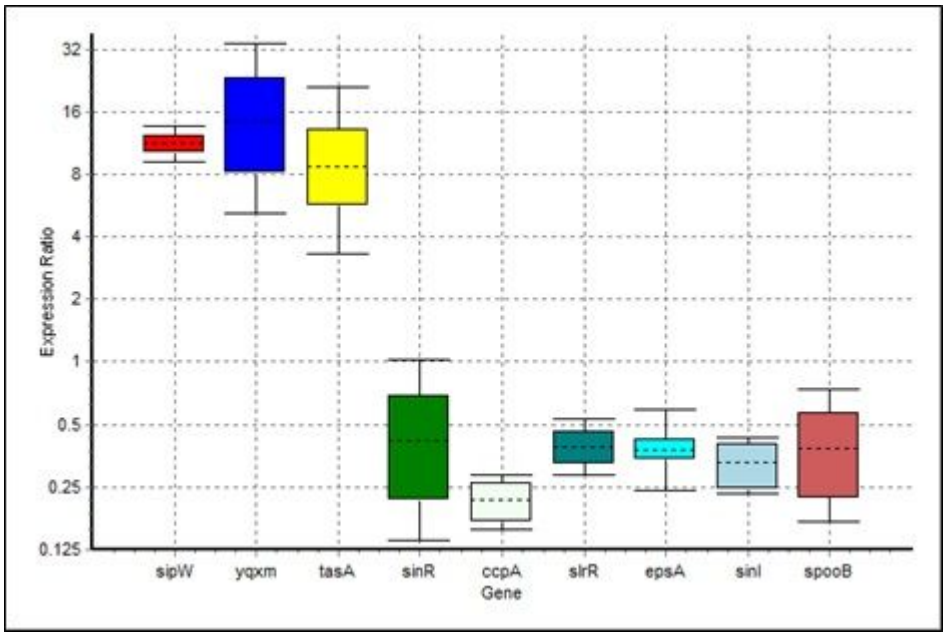

Figure 7

Expression profiling of ionizing-radiation-resistant Bacillus subtilis (RAM-04). The diagram shows a significant over-expression in three genes (YqXm, sipW and tasA known as tasA operon) and shows a significant down-regulated profile of slrR and ccpA genes. This analysis was performed using REST2009V2.0.13 software with $p \leq 0.05$