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Original Article

The Impact of Overexpression of Sigma Factors on Morphological Changes, Growth Pattern, and Biofilm Formation in *Mycobacterium marinum* CCUG 20998

Mohammad Faezi Ghasemi*, Fatemeh Alikhani

Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Lahijan, Iran

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Introduction: Bacteria have at least one sigma factor (σ-factor) that transcribes the genes required for cell viability. Usually, transcription of σ-factors occurs and changes in response to a variety of environmental stresses. Expression of σ-factors is one of the strategies which is used in response to different stress conditions. This study was aimed to evaluate the effects of overexpression of σ-factors genes including σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L, and σ^M on morphology, growth pattern and biofilm formation in *Mycobacterium marinum* CCUG 20998. **Methods**: In this study, the genes for major σ-factors were cloned in the expression vector pAGHD1, containing 11 kb *Hind III* fragment of pAG1 and Tetz determinants. A quantitative real-time PCR (qRT-PCR) assay was used to quantify σ-factor mRNA levels of σ-factors in exponential and stationary phases. The overexpression in real-time experiments was normalized to the σ^A expression level. The effect of expression was evaluated on biofilm formation in this bacterium. **Results:** Some selected σ-factors used in this study were overexpressed. The σ^B had the highest expression level during the exponential and stationary phases. The σ-factors σ^D, σ^G, and σ^H showed lower expression level compared with σ^E, σ^F, and σ^K. The lowest expression belonged to σ^L and σ^M σ-factors. Also, overexpression of σ^E and σ^K led to more biofilm formation in comparison with other σ-factors in *M. marinum* CCUG 20998. **Conclusion:** The overexpression of some σ-factors can affect growth, morphology and biofilm formation in *M. marinum* CCUG 20998. *J Med Microbiol Infec Dis, 2016, 4 (3-4): 68-75.*

Keywords: *Mycobacterium marinum* CCUG 20998, Overexpression, Sigma Factors, Biofilm.

INTRODUCTION

Bacteria have at least one essential sigma factor that transcribes the genes required for cell viability. Sigma factors regulate housekeeping and virulence genes in bacteria [1]. The σ^{70} and σ^{54} families are the two essential σ-factor families [2] that have been identified in the eubacteria. Based on the phylogenetic relationship, the σ^{70} family can be divided into four groups; this division is according to the occurrence or lack of specific regions; group 1 σ-factors contains several specific regions, while group 4 σ-factors group has the lowest number of this regions. These specific regions in groups 2 and 3 show an intermediate frequency. Group 4 σ-factors is also known as an extra cytoplasmic (ECF) σ-factors [3]. The number of σ-factors that correlates with variable environmental encounters vary in different species. For example, *Escherichia coli*, *Streptococcus pyogenes* and soil actinomycetes (*Streptomyces coelicolor*) have 7, 2 and 60 σ-factors, respectively [4]. Moreover, the number of σ-factors varies in *Mycobacterium* spp. [5]. *Mycobacterium smegmatis* and *Mycobacterium leprae* as obligate pathogens have 28 and 4 σ-factors, respectively [2]. *Mycobacterium tuberculosis* has 13 σ-factors; while the *Mycobacterium marinum* M-strain has 18 σ-factors which belong only to the σ^{70} family. The expression and activity of σ-factors are dependent on the growth stage of bacteria and the environmental conditions. There are many reports on overexpression of σ-factors in bacterial species. Overexpression of σ^A enhances *M. tuberculosis* growth in macrophages [6]. The growth of *Corynebacterium glutamicum* decreases upon the overexpression of σ^D or σ^H, but overexpression of σ^H increases the expression of riboflavin biosynthesis genes in *C. glutamicum* [6]. Genetically, *M. marinum* is closely related to *M. tuberculosis* (85% similarity in amino acids and 99.3% in 16S rRNA sequence) and *Mycobacterium ulcerans* in pathogenicity and nucleotide sequences, (99.6% similarity in amino acids) [7]. *Mycobacterium marinum* is an attractive model for the study and identification of infection factors, disease development and drug-resistance of *M. tuberculosis* [8]. In contrast to *M. tuberculosis*, *M. marinum* grows with a relatively shorter doubling time, and its growth is limited at higher temperatures, which makes it easier for laboratory studies comparing to *M. tuberculosis* [9].

***Correspondence:** Mohammad Faezi Ghasemi

Department of Microbiology, Faculty of Basic Sciences, Lahijan Branch, Islamic Azad University, Shagayegh St., Lahijan, Iran, P.O. BOX: 1616.

Email: faezi_m@yahoo.com

Tel/Fax: +98 (13) 42222605

In recent years, several significant developments were achieved regarding our understanding of the *in vivo* roles of σ-factors in the *Mycobacterium* spp., but there is not much information on the *in vitro* effects of these factor overexpression on growth and physiology of these bacteria. This study was aimed to evaluate the effects of overexpression of σ-factors genes σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L and σ^M on the morphology, growth pattern, and biofilm formation in *M. marinum* CCUG 20998.

MATERIAL AND METHODS

Bacterial growth. *Mycobacterium marinum* CCUG 20998 was cultured in Difco™ Middlebrook 7H9 broth, supplemented with 10% (v/v) Middlebrook Oleic Albumin Dextrose Catalase Growth supplemented (OADC), 0.025% (v/v) Tween 80, and 0.4% (v/v) glycerol (prior to autoclave), in rotary at 30ºC and 100 rpm for 7 days. For exponential and stationary phases determination, a single colony of *M. marinum* CCUG 20998 was grown on Middlebrook 7H10 agar plate and inoculated into Middlebrook 7H9 broth (Difco). The cultures were incubated until the cell density reached 0.6-0.8 at the OD_{600} (exponential phase) and early stationary phase (12 h after OD stabilized at 3.5-4.5).

Sigma factors and plasmid construction. Ten genes that produce major σ-factors including σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L and σ^M were amplified by PCR as described by others [10]. These factors were successfully cloned in the expression vector pAGHD1 that contained 11 kb *Hind III* fragment of pAG1 and Tetz and hygromycin resistant gene determinants. The *tetRO* region from TetZ was amplified by PCR using the primers TetR For (5′-CGGGATCCTCACGATTCGCTCGAGGTC-3′) and: TetR Rev (5′-CGGGATCCAGTTGCACTTTATCATCGAT-AAC-3′); moreover, *tetO* determinant was amplified using TetOFor (5′-CGGGATCCAGTTGCATTTATCATCGAT-AAC-3′) and TetORev. The forward primers contained a *NdeI* restriction site [11] and were designed in front of *lacZ* gene to be able to evaluate the induction using beta galactosidase activity assay.

Preparation of electrocompetent and transformation of *M. marinum* cells. *Mycobacterium marinum* cells were cultured in 10ml 7H9 broth supplemented with 10% (v/v) OADC and 0.025% (v/v Tween 20 and incubated in a rotary shaker incubator at 100 rpm at 30ºC for 7 days. The culture was then diluted (1/100 dilution) and incubated for more three days. Upon reaching the cells into the exponential phase (OD_{600}=0.8), cultures were placed on ice for 1.5 h and centrifuged at 13000 g for 10 min. The cell pellet was washed three times using 10% glycerol, resuspended in 1-2 ml 10% glycerol, transferred into cold tubes (200 µl, in each tube) and placed in liquid nitrogen until used. For electroporation, 2 µl pAGHD1 plasmid containing σ-factors was inoculated into 200 µl *M. marinum* competent cell. Then the cells were transferred into 0.2 cm, electrode gap electroporation cuvette (Thermo Scientific, USA). The cuvette was electroporated using a Gene Pulser Xcell™ (Bio-Rad, USA) with the settings, 2.5kV, 25µF, and a1000Ω resistance. For selection, the cells were suspended in 1mL7H9 medium containing 25 μgml^{-1} hygromycin.

Expression of σ-factors. *Mycobacterium marinum* CCUG 20998 cultures were grown to mid-log phase, and then tetracycline with at the concentration of 40 μgmL^{-1} was added to half of the cultures after 72 h of incubation. The cells were collected by centrifugation at 13000 g for 15 min. The pellets were resuspended in 200 µL 10mM Tris-HCl pH 8.0 and transferred to the tubes containing 100 µL 0.1mm silica beads (MP Biomedical, USA). The cells were disrupted using Fast Prep FP120 bead beater (MP Biomedical, USA) for 45 s at the speed 6500 strokes/min. Then the cells were chilled on ice and centrifuged at 13000 g for 3 min. The supernatant was transferred to new tubes and stored at -20°C. Amounts of 15 µL of the sample lysate was added to a 96-well plate, 145 µL 1M KH_2PO_4pH 7.5 buffer containing 250 ml KH_2PO_4 was added to each well. A final concentration 5 $mmolL^{-1}$of chlorophenol red-β-D-galacto-pyranoside (CPRG) (0.05 $molL^{-1}$ KH_2PO_4 and 0.001 molL-^{1} $MgCl_2$) solution was added to each well. The OD_{600} was measured every 15 min up to 90 min.

Isolation of RNA for quantitative real-time PCR (qRT-PCR). The total amount of RNA was extracted from *M. marinum* grown in 7H-9 broth medium (OD_{600} 0.5). The cells were harvested by centrifugation at 13000 g for 1 min, washed in 1 ml GTC buffer [5 M guanidine thiocyanate, 0.5% (w/v) Sarkosyl, 0.5% (w/v) Tween 80, 100 mM 2-mercaptoethanol, 25 mM sodium citrate (pH 7)], and resuspended in 1 ml Trizol reagent (Invitrogen). Then cells were disrupted with 0.5 ml silica beads (0.1 mm diameter) through using Fast Prep FP120 bead beater (MP Biomedical, USA), and RNA was isolated according to the directions for the Trizol reagent. The extracted RNA was suspended in 50 ml DNase buffer [20 mM Tris/HCl(pH 7.5), 10 mM $MgCl_2$], and 20 U RNase-free DNase I (Roche, Switzerland) was added, followed by incubation at 37°C for 1 h. Purification of RNA was performed through using RNeasy kit spin columns (Qiagen, Korea) according to the provided instructions. The RNA was quantified using a Nano Drop ND-1000 spectrophotometer. The RT-PCR reactions were performed in ECO™ Real-Time PCR System (Illumina, USA) using the primers listed in Table 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 10 mL 10% separating gel contained 4.05 mL dH_2O, 3.3 mL 30% acrylamide/bis 37.5:1 (Bio-Rad, USA), 0.1 mL 10% APS and 10 µL (Bio-Rad, USA) TEMED (N, N, N, N-Tetramethylethylene-diamine) (Bio-Rad, USA). The 5 mL 5% stacking gel contained 3.5 mL dH_2O, 0.625 mL 8X stacking buffer (0.92 M Tris-base pH 6.8 and 0.8% SDS), 0.83 mL 30% acrylamide/bis 37.5:1 (Bio-Rad, USA), 0.05 mL 10% APS, and 5 µL TEMED. Amounts of 10 µL protein samples were mixed with 10 µL 2x SDS loading buffer containing 125 mM Tris-HCl pH 6.8, 5% SDS, 25% glycerol, and 0.05% bromophenol blue. The protein samples were boiled at 95ºC for 3 min before loading on 10% one-dimension running gel. Molecular weight marker and Page Ruler plus prestained protein ladder (Fermentas, USA) were run along with the samples.

Estimation of biofilm formation. Biofilm formation was evaluated through using Carter's method [12, 13] with some modifications. Amounts of 100 µl of frozen stock culture were inoculated in 10 ml 7H9 medium with OADC and Tween 80. The incubation was performed at 30º C with agitation for seven days. An amount of 200 µl of the cell suspension was added to the wells of a 96- well flat bottom polystyrene micro titer plate (MicroWell™ Plates Nunclon™ NuncNuncleon, Roskilde, Denmark) and incubated at 30ºC without agitation in a sealed container with 20 ml sterile distilled water to prevent drying. Culture plates with no bacteria were included as negative controls. After incubation for two weeks, bacterial growth was determined by the measurement at OD_{600}. The wells were washed with 250 µl of tap water, and the remaining biofilm was stained with 250 µl of 1% crystal violet and incubation for 30 min at room temperature. The wells were washed three or four times with tap water. The stained biofilm was suspended in 250 µl of a mixture of ethanol and acetone (70:30). Finally, the biofilm formation was measured at OD_{600}. Results were presented as the median value of triplicate with subtracting the average value for the negative control. Statistical analysis were performed using spss software v 23.0.

Table 1. Primers for qRT-PCR analysis

Primers	Sequence	Primers	Sequence
Sigma A-qRT-fw	GCACCACCGGCGCAC	**Sigma G -qRT-rv**	CCTAAGGCCAAAGGCCTC
SigmaA- qRT-rw	ACTGGGCCCAGCTTGTC	**Sigma H-qRT-fw**	GCGTGGCTGTACGGGATT
Sigma B-qRT- fw	AGCCCTGCAGCGGACTT	**Sigma H-qRT-rv**	CGCTGCTTCTTGCGATAGCT
Sigma B-qRT- rv	TTGACCAACGGGGTCTTG	**Sigma J-qRT-w**	GCGAACTAACCGGCGTCTCA
Sigma D-qRT- fw	CAGTCCGGTCTCAGCAGA	**Sigma J-qRT-rv**	CCAGTGTGTACCAGGGCAATAC
Sigma D-qRT- rv	CCGAAATCCTGGCCTTAC	**Sigma K-qRT- fw**	AGGCGTTCCGGATGAATCT
Sigma E- qRT- fw	GACGTTCTGCCGATATTGA	**Sigma K-qRT- rv**	GTGCCAGGAGTCCATACACTAA
Sigma E -qRT- rv	AAGGTCCTGATCGGGTGTTCC	**Sigma L-qRT- fw**	CCAAGAAACACCCGCTCTA
Sigma F -qRT- fw	GCGTGGCTGTACCGGATT	**Sigma L-qRT- rv**	ATCCCGTAGCGCCACAACATTA
Sigma F- qRT- fw	ACCGGTTCACCTTGTTCTTCT	**Sigma M-qRT-fw**	ACACCCACAACGGCTCAA
Sigma G -qRT-fw	GAGACCCATGTGCCTTTTCAG	**Sigma M-qRT-rv**	GACACCGCATTGCAATTGGCCG

RESULTS

In this study, the overexpression of eleven major σ-factors in *M .marinum* CCUG 20998 was examined through using pAGHD1 vector. Figure 1 shows the microscopic view of the growth patterns of *M. marinum* CCUG 20998 transformant cells containing 11 σ-factors σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L and σ^M compared with the wild type strain in 7H-9 medium. The σ^E led to more clump formation of *M. marinum* cells (Fig. 1). The transformants containing σ^J and σ^F showed a reduction in clump formation and the cells tended to assume planktonic forms more than the others (Fig. 1 J, I and L). The cells containing σ^B, σ^E and σ^K formed more clumps in the culture media. Both clumping and planktonic forms were seen in the wild type strains that had grown in 7H9 broth (Fig. 1A). *Mycobacterium marinum* cells transformed with σ^E and σ^K constructs showed a poor growth in 7H9 broth and formed biofilm in flask cultures. The Estimation of generation time for σ^E and σ^K constructs showed an increase of ≈ 4-6 h, and the generation time reached to 8-12 h. To determine the pattern of tetracycline induction, the growth of *M. marinum* in 7H9 broth was allowed to continue for 10 days and β-galactosidase activity was measured. The results indicated that β-galactosidase activity reached a plateau within 7 days of induction. After 4 days of induction, a significant increase in enzyme activity was seen until reaching a plateau in the day 7. Upon removal of tetracycline, a decrease in β-galactosidase activity was observed, and the enzyme activity returned to the basal level. The maximum β-galactosidase activity in *M. marinum* CCUG 20998 containing 11 different annotated σ-factors is depicted in Figure 2. As shown, the maximum β-galactosidase activity was observed in the constructs containing σ^A and σ^B, equivalent to about 18 and 12 U/mg proteins, respectively. The obtained result indicated that the overexpression of σ^B had more effect on the production of β-galactosidase than the other σ-factors. The σ-factors σ^D, σ^G, σ^H, σ^J were expressed at a lower rate than σ^E, σ^F, and σ^K, and the lowest expression rate belonged to σ^L and σ^M. To validate these findings, quantitative real-time PCR (qRT-PCR) was used to quantify σ-factor mRNA levels of *M. marinum* constructs at the exponential and stationary phases. The level of mRNA for each σ-factor in the exponential and stationary phases relative to σ^A estimated by the qRT-PCR is shown in Figures 3 A and B. The (qRT-PCR) results revealed that σ^B transcript was higher than the other σ-factors in both phases. The lower transcripts were identified in constructs with the σ^D, σ^G, σ^H, and σ^J. The quantity of σ^E, σ^F and σ^K were at the middle, and σ^L and σ^M were expressed at the lowest level as shown in Figure 3 A and B. The level of σ^F expression was more in the stationary phase in comparison to the exponential phase (the increase of overexpression was statistically significant p=0.047). The amount of each σ-factors transcripts was calculated relative to the σ^A transcripts (the increase of overexpression was statistically significant p=0.048). These results have been in agreement with the obtained results for β-galactosidase activity. The SDS-PAGE of the cell extracts of *M. marinum* containing eleven σ-factors σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L and σ^M is shown in Figure 4. The estimated molecular weight for σ^A was 58 kDa, σ^B 23 kDa, σ^D 35 kDa, σ^E 48 kDa, σ^F 26 kDa, σ^G 41 kDa, σ^H 37 kDa, σ^J 30 kDa, σ^K 42 kDa, σ^L 50 kDa and σ^M44 kDa. The overexpression of σ^E and σ^K led to more biofilm formation in comparison to other σ-factors in 7H9 broth with Tween 80 incubated at 30ºC as shown in Figure 5. The Biofilm formation by σ^B, σ^E and σ^K was about 50% more than the other σ-factors.

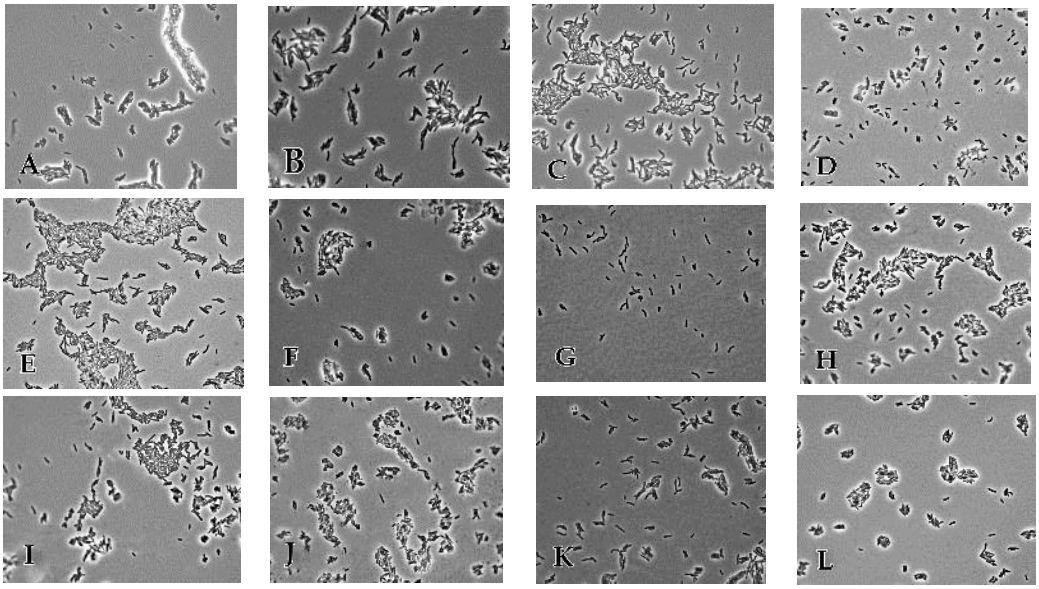

Fig. 1. Phase contrast microscopic views of the constructs containing different σ-factors. A wild type; B: σ^A, C: σ^B, D: σ^D, E: σ^E, F: σ^F, G: σ^G, H: σ^H, I: σ^J, J: σ^K, K: σ^L and L: σ^M. Samples of each σ-factor culture were collected at the same time, and the selected image for each σ-factor is an average of 20 microscopic fields of view, using Axioplan 2 phase contrast microscope (Carl Zeiss, Germany).

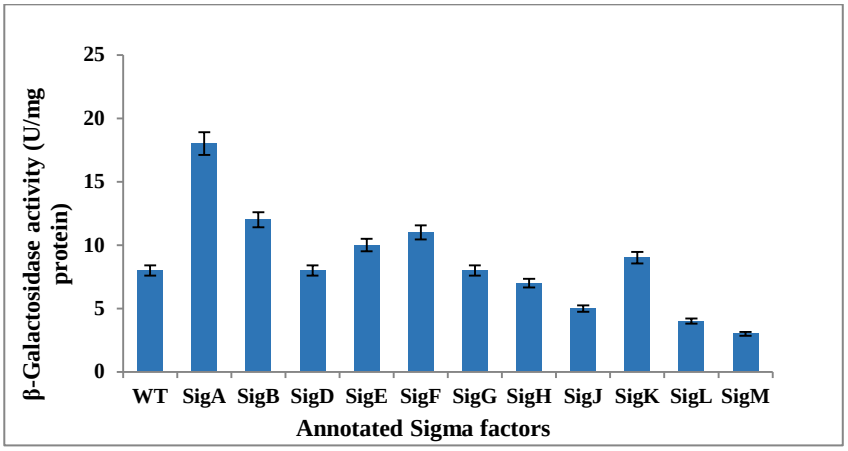

Fig. 2. The β-galactosidase activity of *M. marinum* CCUG 20998 containing 11 different annotated σ-factors. The results are given as means±SD of three replicates for each σ-factor.

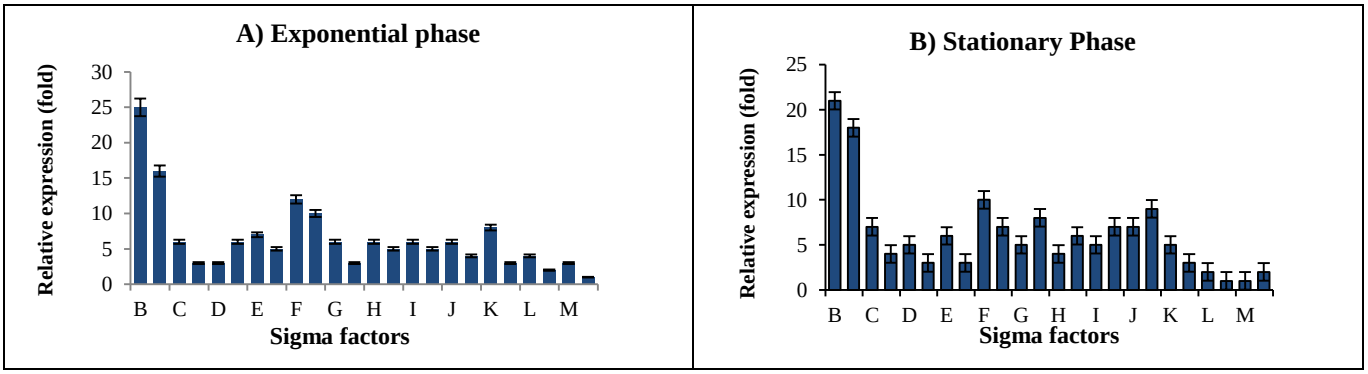

Fig. 3. The level of mRNA overexpression of the σ-factors in *M. marinum* CCUG 20998 estimated by qRT-PCR. A is exponential and B stationary phase in 7H9 medium. The Ct value level of expression of σ-factors normalized to that of Sig A, and the relative expression was calculated by the ΔΔ Ct method compared to the control strain

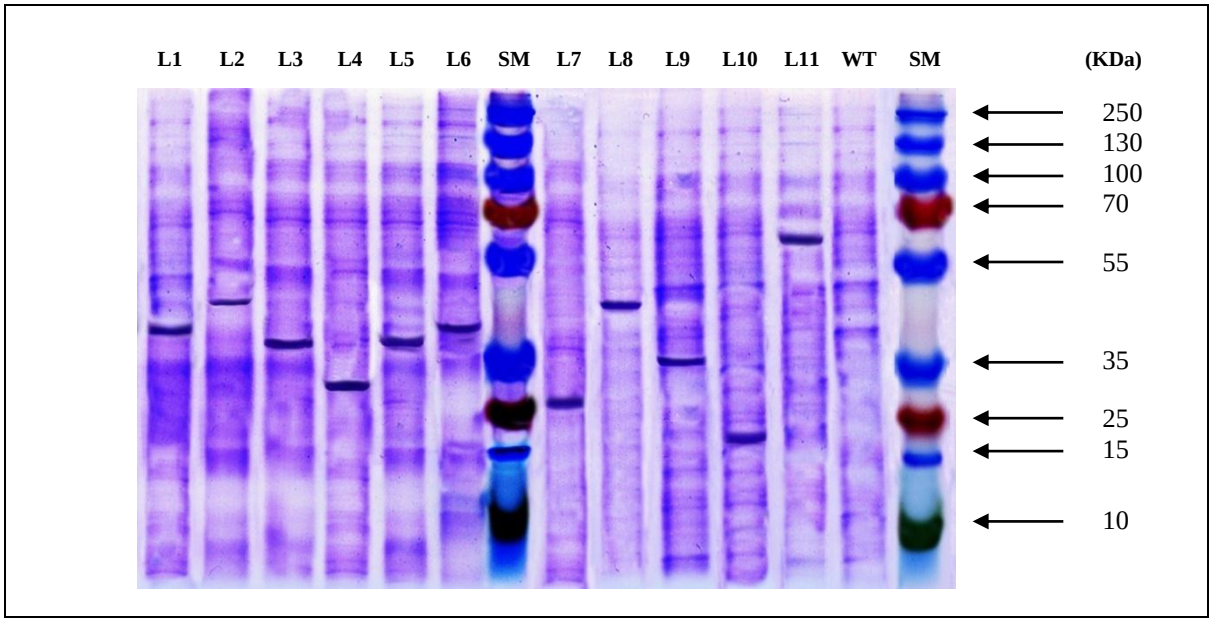

Fig. 4. SDS-polyacrylamide gel electrophoresis of cell extracts of *M. marinum* containing 11 σ-factors (σ^A, σ^B, σ^D, σ^E, σ^F, σ^G, σ^H, σ^J, σ^K, σ^L and σ^M). Lane 1, (σ^M 44 kDa); lane 2, (σ^L 50 kDa); lane 3, (σ^K 42 kDa); lane 4, (σ^J 30 kDa); lane 5, (σ^H 37 kDa); lane 6, (σ^G 41 kDa); size marker (SM), lane 7, (σ^F 26 kDa); lane 8, (σ^E 48 kDa); lane 9, (σ^D 35 kDa); lane 10, (σ^B 23 kDa); lane 11, (σ^A 58 kDa); wild type (WT) and size marker (SM).

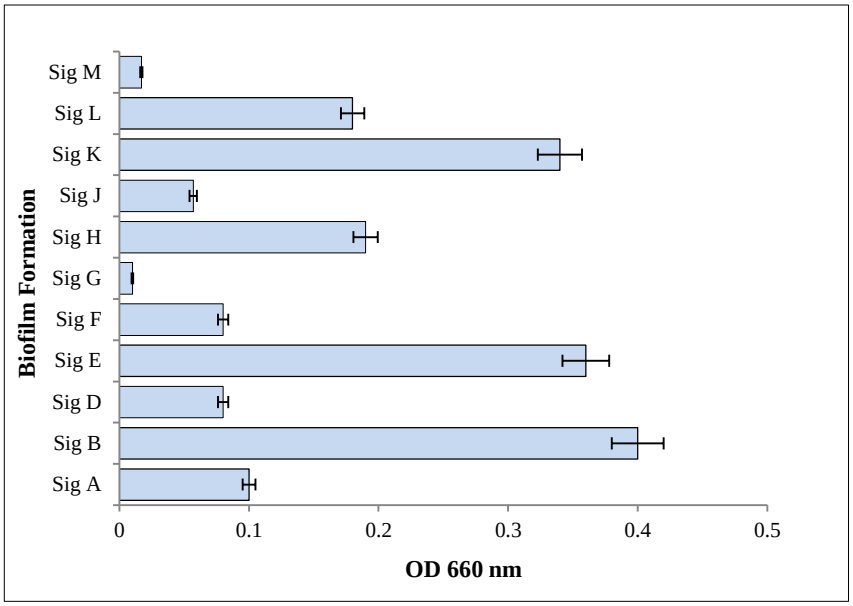

Fig. 5. Biofilm formation by *M. marinum* CCUG 20998 containing different σ-factors; Results are means ± SD of triple determination for each σ-factor.

DISCUSSION

Sigma factors in RNA polymerase (RNAP) of eubacteria give promoter selectivity, enabling the transcription pattern to change in response to the environmental changes [14]. The ability of *Mycobacteria* spp. to adapt to the environmental changes is mediated through its transcriptional elasticity by the σ-factors. Among the species of the genus *Mycobacteria*, there is more information regarding *M. tuberculosis*. This bacterium encodes at least 13 σ-factors [15, 16]. *Mycobacterium marinum* has a large genome with the high capacity to survive in different environmental conditions; it also possesses (a) a single circular chromosome that is rich in GC (62.5%) which contains 6.638.827 base pair, (b) 5424 predicted coding sequences (CDS), (c) 65 pseudogenes, (d) 46 tRNA one single rRNA operon, and (e) a 23 kb plasmid designated as pMM23. In contrast to *M. tuberculosis*, which has no environmental reservoirs, *M. marinum* is found in various aquatic environments including swimming pools and drinking waters [17-19]. In

this study, overexpression of eleven σ-factors in *M. marinum* CCUG 20998 was investigated using pAGHD1vector. All the selected σ-factors that were utilized in this study showed overexpression; however, the levels of expression were different in the exponential and stationary phases. Overexpression of σ^B was more significant than other σ-factors during exponential and stationary phases. The transcript of σ^E was more abundant in the stationary phase and upon aging of the cells. The overexpression of σ^A, σ^B, and σ^E had a significant effect on the growth pattern, clumping, and biofilm formation of *M. marinum*, especially for the constructs containing σ^B and σ^E. It has been shown that σ^A, σ^B, and σ^E are most abundant *in vivo* conditions. For instance, overexpression of σ^A enhanced the growth of *M. tuberculosis* H37Rv in human macrophages; suggesting its role in virulence [6]. The σ^B is involved in the general stress response in *M. tuberculosis*, *M. smegmatis*, and *M. marinum* and is similar to the C-terminal portion of σ^A; moreover, the regulons of σ^A and σ^B do not overlap much, except for a few genes [20]. Five σ-factors, σ^D, σ^F, σ^G, σ^H, and σ^J are present in the genomes of all species of the genus *Mycobacteria*, except *M. leprae*. Among these σ-factors, overexpression of σ^D was not significant with the pAGHD1 vector in the stationary and exponential phases and did not affect the growth pattern. Usually, σ^D is overexpressed during nutrient starvation [21], and infection with *M. tuberculosis* decrease the intracellular level of σ^D. The loss of σ^D did not affect the ability of *M. tuberculosis* in macrophages [22, 23]. In this study expression of σ^E, σ^F and σ^K is increased in constructs containing the vector in both exponential and stationary phases. Also, results of this study showed overexpression of σ^E and σ^K led to more biofilm formation by *M. marinum*. The expression of σ^E increased following exposure to heat and growth in mononuclear phagocytes. In *M. smegmatis*, σ^E was not essential for growth, and its deletion caused increased susceptibility to oxidative stress and acidic pH [24]. Hence, it can be concluded that σ^E transcription is effective in stress conditions. In this study, modification of cell envelope in *M. marinum* changed by the nutrient availability, pH and osmolarity, which was involved in the attachment of bacteria to different surfaces and biofilm formation. The expression of genes involved in the biosynthesis of complex polysaccharides, lipids, sulfolipids, energy metabolism and nucleotide synthesis is under the σ^F control [25]. More biofilm formation is due to the overexpression of σ^F, which increased the biosynthesis of cell envelope polysaccharides in the stationary phase of growth of *M. marinum*. Overexpression of σ^F during exponential growth phase in *M. tuberculosis* neither resulted in any growth arrest nor reduced the susceptibility to rifampin and isoniazid antibiotics [25]. Also, σ^F mRNA level was higher in stationary growing *M. tuberculosis* and upon exposure to oxidative stress [10]. According to our results σ^K seems to have an additional role in the biosynthesis of cell envelope materials, which leads to more biofilm formation in *M. marinum*. The σ^G is one of the first induced genes in *M. tuberculosis* during macrophage infection and when this bacteria encounters stress conditions such as heat and cold shocks as well as low aeration, [26]. Also, σ^G is induced by DNA damage response in *M. tuberculosis* [27]. Upon vector transformation containing σ^G, the expression level of σ^G did not change, and there was not any significant effect on morphological, growth pattern, and biofilm formation in *M. marinum* CCUG 20998. The σ-factors σ^H, σ^J, σ^L and σ^M are overexpressed in responses to different stresses. The σ^H expressed upon exposure to oxidative and heat stresses in *M. smegmatis* and *Mycobacterium avium* ssp. *Paratuberculosis* [28] and σ^J genes are expressed in late stationary phase in the dormant cultures of *M. tuberculosis* [29]. More expression of σ^H, σ^J was detected in the exponential phase of *M. marinum* in 7H9 medium. The biofilm formation was more in the cells containing pAGHD1 expression vector in contrast to the controls. Moreover, the level of σ^J mRNA increases after the heat and cold stresses in *M. marinum* cells grown on 7H10 medium [10]. In this study, σ^L and σ^M were expressed at low level in the exponential phase of growth. The overexpression of σ^M induces in stationary phase and high temperatures in *M. smegmatis*, *M. bovis* BCG, and *M. tuberculosis* CDC1551 [30]. The overexpression of other σ-factors such as σ^G, σ^H, σ^M and σ^J increases over time in response to cold and heat stresses in *M. marinum* [10]. A mutation in σ^L exhibited marked attenuation in virulence of *M. tuberculosis*, suggesting a role of this σ-factor in virulence [31]. The overexpression of σ-factors in the genus *Mycobacterium* depends on several factors. So, expression of different σ-factors in the genus *Mycobacteria* in various growth stages and various conditions of growth are useful in determining their functions and obtaining valuable information regarding the genes controlled by σ-factors. The results of this study indicated that σ-factors could affect growth, morphology and biofilm formations in *M. marinum*. It has been shown that *M. marinum* binds to a variety of abiotic surfaces under different conditions. The cell wall components mediate the initial attachment and expression of σ-factors can affect this attachment, especially in aquatic environments, where the bacteria can form biofilms or grow as planktonic cells [17]. The expression of the σ-factors in *Mycobacterium* spp. depends on several factors. Under stress conditions and during host infection, levels of mRNA for σ-factor are changed in exponential and stationary phases [25]. To understand the expression and the impact of σ-factors on growth and the physiology of *Mycobacterium* spp., post-transcriptional regulation should be studied. The factors such as mRNA stability, promoter organization and translational start sites, chromosomal location and organization, and impact of anti-σ-factors are important and need to be more investigated in members of the genus *Mycobacterium*. Also, studies on overexpression of other five σ-factors including σ^{C-1}, MMAR0975 (σ^{0975}), MMAR3276 (σ^{3276}), MMAR3687 (σ^{3687}), MMAR4487 (σ^{4487}) will provide more information regarding their effects on morphology, growth pattern, and biofilm formation in *M. marinum* CCUG 20998. The biofilm formation by *M. marinum* CCUG 20998 under different stress conditions showed that biofilm formation reduced about 90% at pH 11 and hydrogen peroxide at a concentration of 9600 ppm [13].

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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