The sigma factor SigD of Mycobacterium tuberculosis putatively enhances gene expression of the septum site determining protein under stressful environments

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SUMMARY

This work examined the expression of the septum site determining gene (ssd) of Mycobacterium tuberculosis CDC1551 and its ΔsigD mutant under different growing conditions. The results showed an up-regulation of ssd during stationary phase and starvation conditions, but not during in vitro dormancy, suggesting a putative role for SigD in the control of ssd expression mainly under lack-of-nutrients environments. Furthermore, we elucidated a putative link between ssd expression and cell elongation of bacilli at stationary phase. In addition, a -35 sigD consensus sequence was found for the ssd promoter region, reinforcing the putative regulation of ssd by SigD, and in turn, supporting this protein role during the adaptation of M. tuberculosis to some stressful environments.

About one-third of the world’s population is infected with Mycobacterium tuberculosis (Mt) in a dormant stage (latent infection) (Korb et al., 2016). During dormancy, the bacilli establish a non-replicating state thought being related to hypoxic and nutrient starvation conditions (Dietrich et al., 2015; Sershen et al., 2016). Adaptation of the bacilli to active duplication or to dormancy requires a tight regulation of gene expression. To allow that adaptation, the Mt genome encodes 13 putative sigma factors, which control gene expression in response to specific extracellular signals. Some sigma factors act as housekeeping genes but others, called alternative sigma factors, have been identified as regulators that respond to specific environmental stimuli and stressors (Manganelli et al., 2004; Chauhan et al., 2016).

The extracytoplasmic alternative sigma factor SigD of Mt controls the expression of some ribosomal protein genes whose expression is normally induced during the stationary growth phase in vitro, suggesting that SigD activity is expressed late in the growth cycle and that it acts as an alternative sigma factor mediating stationary phase homeostasis (Calamita et al., 2005). Furthermore, SigD expression was induced under the starvation model of M. tuberculosis persistence, suggesting it could be important for adaptation of the bacilli during nutrient limitation (Betts et al., 2002). Moreover, it has been demonstrated in a mouse infection model that an attenuated Mt sigD mutant strain showed significant differences in survival, such as a decreased replication in lung and spleen tissues (Raman et al., 2004). In Mt, cell division regulation occurs by the polymerization inhibition of protein FtsZ in response to stress conditions (Slayden et al., 2006). The septum cell site selection and regulation are controlled by MinC, MinD and MinE proteins and some other septum site determining proteins, which together negatively regulate FtsZ polymerization, which in turn, stops cell division (Slayden et al., 2006; Lutkenhaus, 2007). The MT3760 gene of M. tuberculosis CDC1551, homologue of the Rv3660c gene of M. tuberculosis H37Rv, codes for the septum site determining protein (Ssd) that promotes bacterial filamentation by inhibition of septum synthesis, leading to elongated cells as a response to a stressful environment (England et al., 2011). In addition, Rv3660c is the first component of a genomic island that codes for the Mt type IV pili (Ramsugit et al., 2015), which is involved in the assembly and secretion of such cellular structure (Tomich et al., 2007; O’Connell et al., 2011; Perez-Cheeks et al., 2012). In order to investigate whether SigD affects ssd regulation in a stressful envi-
enronment, possibly related to the slow-down division of the bacteria and pili assembly, we determined the ssd expression of the *M. tuberculosis* CDC1551 ΔsigD mutant strain during stationary phase, and hypoxic and nutrient starvation conditions.

*M. tuberculosis* CDC1551 wild-type and ΔsigD strains (Lamichhane et al., 2003) were cultured in Middlebrook 7H9 medium (Difco) supplemented with 10% ADC (albumin, dextrose and catalase enrichment; BBL) at 37°C. Growth kinetics of wild-type and ΔsigD strains were performed from bacterial cultures growing for four weeks and optical density values at 600 nm (OD<sub>600</sub>) were measured every 24 h. Cells were harvested at exponential (OD<sub>600</sub>=0.4) and stationary (OD<sub>600</sub>=1.2) phases.

For the hypoxia conditions exponentially growing cultures were subjected to a microaerophilic environment, termed a non-replicative persistence 1 (NRP1) stage, and to an aerobic environment, termed a non-replicative persistence 2 (NRP2) stage, as described by Wayne and Hayes (1996). For the nutrient starvation conditions exponentially growing cultures were pelleted and then cells were washed twice and resuspended in PBS 1X. Cultures were incubated at 37°C and harvested at 24 h and 96 h according to Betts et al. (2002).

RNA extraction was performed as previously described (González-y Merchand et al., 1996). The quality of RNA was assessed using a NanoDrop (ND-1000; Thermo Scientific) and the Agilent bioanalyzer. cDNA was synthesized using 1 µg of RNA, 0.2 µl of random hexamer primers and 2 U/µl of M-MulV-RT (Reverse transcriptase of Moloney Murine Leukemia Virus; Thermo Scientific).

Quantitative real-time PCR was performed in a LightCycler 480 instrument (Roche) to quantify the expression levels of *ssd* gene in the wild-type and ΔsigD strains under different growth conditions. We also measured the expression of *sigD* in the wild-type strain as well as the expression of *fixA* and *clpX* (as positive controls) and *lipY* and *rpfB* as (negative controls), according to Calamita et al., 2005. Specific gene primers (Table 1) were designed with the Primer3Plus software (http://www.bioinformati cs.nl/cgi-bin/primer3plus/primer3plus.cgi/) (Untergasser et al., 2012). For LightCycler reactions, a master mix of the following components was prepared: 3.0 µl of PCR-mix, 1.0 µl (10 µM) of forward primer, 1.0 µl (10 µM) of reverse primer, 10 µl of 2x Master Mix and 5.0 µl of cDNA (50-100 ng). A multiwell plate containing all samples was loaded into the LightCycler 480 instrument. Amplification was performed in triplicate wells for each sample analyzed from three independent experiments. In each set of reactions, 16S rRNA was used as a reference gene for normalization of cDNA amount. Real-time PCR analysis was performed using the following optimized assay conditions:

1) denaturation program (95°C for 10 min); amplification and quantification program repeated for 45 cycles (95°C for 10 s, 58°C for 20 s, 72°C for 30 s with a single fluorescence measurement);

2) melting curve program (95°C for 10 s, 65°C for 1 min with continuous fluorescence measurement at 97°C) and finally;

3) a cooling step at 40°C for 10 s.

Absolute quantification was carried out by obtaining standard curves for each set of primers according to 10-fold dilutions of known amounts of *M. tuberculosis* CDC1551 chromosomal DNA. Crossing point values were interpolated to standard curve to obtain the number of gene copies per µg of RNA. For statistical analysis one-way ANOVA followed by the Tukey’s comparison test was performed by using GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA). In all cases, a p<0.05 was considered statistically significant.

For confocal microscopy, *Mtb* wild-type and ΔsigD cultures recovered from exponential and stationary phases of growth were concentrated by centrifugation and stained with Auramine-Rhodamine T (TB Fluorescent Stain Kit T, Becton Dickinson, Sparks, MD, USA) as previously described (McCarte and Robinson, 1994). These cultures were then examined by confocal laser scanning microscopy (LSM 5 exciter microscope, Carl Zeiss, Oberko-chen, Germany) using a 100X oil-immersion objective for all magnifications. Scanned samples were analyzed by software (LSM, Zeiss) for image projection. In all cases, 20 fields were evaluated to calculate an average in size of *Mtb* cells.

For the analysis of the upstream region of the *ssd* gene, 400 nucleotides (nt) were taken upstream of the initiation codon. This region was analyzed by using the Genome2D webserver for prediction of prokaryote promoters (http://

### Table 1 - Primers used in this study.

<table>
<thead>
<tr>
<th>Gene (Name, <em>CDC1551</em>)</th>
<th>Sequence (5’ to 3’)</th>
<th>Product name</th>
<th>Reference</th>
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| *ssd* (MT3760)         | F: CTGGGGCGGCTGCCATAA  
                         | R: CACCGCGCACAATTCGA  | Septum site determining protein (*ssd*) |
| *SigD* (MT3523)        | F: TCACCCGAGTAAACAGATG  
                         | R: AGCGACACCGCAAGATGGA  | Alternative sigma factor (*SigD*) |
| *fixA* (MT3113)        | F: TGAATCGAGCGATCCAGATAC  
                         | R: TTCTTTCCTCAGAATCGG  | Electron transfer flavoprotein (*FixA*) |
| *lipY* (MT3181)        | F: GCAATGCGGATATTACC  
                         | R: CCCAGCGATGGGAC  | Triacylglycerol lipase (esterase/lipase, *LipY*) |
| *clpX* (MT2532)        | F: TCTGATCAAGTCTGCCTGAG  
                         | R: TTGACAAAAAGCTTTCTCGG  | CLP protease ATP-binding subunit (*ClpX*) |
| *rpfB* (MT1038)        | F: CGCGAACATAGCCCTCAAGA  
                         | R: CGACCTTCGGGCTCTCAT  | Resuscitation promoting factor (*RpfB*) |
| *rrs*                  | F: GTCATGCGATGCAAGAGAC  
                         | R: TTGCGCGTGTCTCCGTTC  | 16S Ribosomal RNA |

*Data taken from Tuberculist database: www.tuberculist.epfl.ch*
In order to find a possible consensus motif, the predicted promoter was compared with the consensus recognition sequences of the known SigD-dependent genes (sigD, rpfC, Rv1815), using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences were aligned with ClustalX2 (Larkin et al., 2007) and MUSCLE (Edgar, 2004). Subsequently, these were analyzed with the WebLogo software (http://weblogo.berkeley.edu/) to establish nucleotides that are conserved among all sequences (Crooks et al., 2004).

This report found that the in vitro growth rates of the wild-type and ΔsigD mutant strains were very similar in both exponential and stationary phases (Figure 1). These results confirm that absence of sigD does not affect the growth of Mtb as previously reported by Raman et al. and Calamita et al. (Raman et al., 2004; Calamita et al., 2005). We have also shown that the expression of ssd in the wild-type strain of Mtb was 9.4-fold higher in the stationary phase than in the exponential phase (Figure 2a). In contrast, when a Mtb SigD mutant was employed, the ssd expression was similar in both growing conditions, exponential and stationary (Figure 2a). These results are in accordance with the previously reported role of Ssd in modulation of septum formation and cell division when Mtb faces a stressful environment (England et al., 2011). Likewise, it has been demonstrated that these mycobacteria acquired a filamentous phenotype in the stationary phase when fatty acids are used as sole carbon source (Rodríguez et al., 2014), and when bacterial division is inhibited by inactivation of the penicillin-binding protein PtsI (Slayden and Belisle, 2009). Additionally, we can

![Figure 1 - Growth curves of M. tuberculosis CDC1551 wild-type and ΔsigD mutant 347 strains at 37°C. Bacterial cultures were grown for four weeks in Middlebroook 7H9 348 medium supplemented with 10% ADC. OD values were obtained every 24 h.](image1)

![Figure 2 - Expression of M. tuberculosis ssd, sigD, fixA and lipY genes during different stressful conditions. (a) Expression of ssd in the wild-type and ΔsigD mutant strains. (b) Expression of sigD in the wild-type strain. (c) Expression of fixA (positive link to sigD) in the wild-type and ΔsigD mutant strains. (d) Expression of lipY (no link to sigD) in the wild-type and ΔsigD mutant strains. Data represent mean copies per µg RNA of three independent experiments with SD values. 16S rRNA was used as a reference gene for normalization. Statistically significant was considered as follows: ns, not significant, *p<0.05, **p<0.01, ***p<0.001.](image2)
suggest that the ssd gene might be overexpressed in the stationary phase for the possible assembly and secretion of the Mtb type IV pili, as reported for other pathogenic bacteria, such as Pseudomonas aeruginosa (Bernard et al., 2009). This suggested up-regulation of the type IV pili may, in turn, influence the aggregation of cells in the stationary phase allowing Mtb to persist under this environmental condition, as proposed for Xylella fastidiosa and Vibrio parahaemolyticus (Li et al., 2007; Frischkorn et al., 2013).

Furthermore, the level of expression of SigD in the wild-type strain was significantly higher in the stationary phase than in the exponential phase of growth supporting the putative role of SigD in the regulation of ssd expression (Figure 2b). By using the Wayne and Hayes dormancy in vitro model (1996), we have shown that the expression of ssd gene was very similar during both stages of hypoxia (NRP1 and NRP2 phases) (Figure 2a). Moreover, our study also demonstrated that SigD expression is decreased during hypoxic conditions (NRP1 and NRP2 phases) (Figure 2b), as previously reported (Raman et al., 2004; Voskuil et al., 2004). We propose that this diminished production of SigD probably provokes the concomitant decreased of ssd expression during this in vitro model of dormancy remaining similar to that observed during the exponential phase. Since the expression pattern of ssd (during NRP1 and NRP2) in the ΔsigD mutant strain was similar to the wild-type (Figure 2a), we suggest that in the absence of SigD some other regulators may exist which maintain the expression of ssd gene in this particular environmental condition. On the other hand, we found that the Mtb wild-type expression of ssd was considerably higher at 24 h and 96 h of the nutrient starvation conditions (6.2- and 12.1-fold, respectively) than in the exponential phase (Figure 2a). Therefore, our hypothesis is that ssd would be up-regulated in starvation conditions in order for Mtb to survive under precarious environmental states such as lack of carbon and energy sources. This assumption is supported by the results obtained with the ΔsigD mutant strain in which the ssd expression pattern under starvation conditions is very similar to exponential phase (Figure 2a), and by the significant increase in the levels of expression of SigD observed in both starvation conditions with respect to the exponential phase of growth in the wild-type strain (Figure 2b), results that coincide with those reported by Betts et al., 2002. Hence, it seems that levels of SigD expression are increased during stressful conditions, which may be important for the survival of Mtb under nutrient deprivation conditions. This assumption is supported by the results obtained with the ΔsigD mutant strain in which the ssd expression pattern under starvation conditions is very similar to exponential phase (Figure 2a), and by the significant increase in the levels of expression of SigD observed in both starvation conditions with respect to the exponential phase of growth in the wild-type strain (Figure 2b), results that coincide with those reported by Betts et al., 2002. Hence, it seems that levels of SigD expression are increased during stressful conditions, which may be important for the survival of Mtb under nutrient deprivation conditions.
expression are linked to ssd expression under starvation conditions. All gene expression results mentioned above were confirmed by a positive control gene (fixA) reported to be linked to sigD, which showed a lower expression in the mutant strain compared to the wild-type strain, regardless of the growth condition (Figure 2c). The same result was obtained when cpx gene, another positive control, was used (results not shown). In contrast, when a negative gene control (lipY) expression was measured in all conditions, it showed no significant difference between the wild type and the mutant strains (Figure 2d). Again, another negative control gene (rpf/B) showed equivalents results to lipY (data not shown).

In order to evaluate the link between ssd gene expression and cell length (as a measure of cell elongation) we performed confocal microscopy of Mtb wild-type and ∆sigD cells stained cultures (harvested from exponential and stationary phases) with Auramine-Rodamine. We found that ∆sigD strain showed a similar length with respect to the wild-type strain in the exponential phase (Figures 3a and 3b). Interestingly, mutant bacilli presented a smaller cell length than wild type strains at stationary phase (Figure 3d). This may suggest that the absence of sigma factor SigD decreases elongation of bacilli at the stationary phase due to the down-regulation of ssd. These observations also correlate with the gene expression data shown in Figure 2a. We suggest that these findings should be further investigated using other stressful conditions.

To investigate whether the ssd gene could be regulated at the transcriptional level by SigD, we searched for a possible consensus motif in the region corresponding to 400 nt, upstream of the initiation codon. We identified a DNA motif of 28 nt at position -315. According to the known -35 SigD consensus recognition sequence GTAACGcT of the promoters previously identified for Mtb (Raman et al., 2004), we found a probable -35 consensus region in the predicted promoter for the ssd gene (Figure 4a). When this sequence was analyzed with the WebLogo software (Larkin et al., 2007), the -35 region showed high similarity with the rest of sequences already reported (Figure 4b), suggesting that the predicted sequence for the promoter of the ssd gene may have a potential consensus motif to be recognized by the sigma factor SigD.

In summary, we found that the expression of the ssd gene in Mtb is increased in the stationary phase and starvation conditions. This phenomenon may be controlled by sigma factor SigD. We have also shown a possible link between ssd expression and cell length of Mtb. As a consequence, SigD could produce a down-regulation of cell division and possibly an up-regulation of synthesis of the type IV pilus of Mtb. These findings could encourage researchers to further investigate cellular growth control of Mtb when it faces stressful environments. These data may shed further light on the host-pathogen relationship of TB.

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Conflict of Interest
There is no conflict of interest to declare for the publication of this paper.

References


