New Microbiologica, 40, 3, 199-204, 2017, ISN 1121-7138

SHORT COMMUNICATION

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 Δ*sigD* 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.

Received August 18, 2016 *Accepted* May 15, 2017

About one-third of the world´s population is infected with *Mycobacterium tuberculosis* (*Mtb*) 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 *Mtb* 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 *Mtb* controls the expression of some ribosomal protein genes whose expression is normally induced during the stationary growth phase *in vitro,* suggesting that Sig*D* 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 *Mtb sigD* mutant strain showed significant differences in survival, such as a decreased replication in lung and spleen tissues (Raman *et al*., 2004). In *Mtb,* 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 *Mtb* 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-

Key words:
SigD, Septum site determining protein, Type IV pili, Stressful environments, M. tuberculosis.

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ronment, possibly related to the slow-down division of the bacteria and pili assembly, we determined the *ssd* expression of the *M. tuberculosis* CDC1551 Δ*sigD* mutant strain during stationary phase, and hypoxic and nutrient starvation conditions.

M. tuberculosis CDC1551 wild-type and Δ*sigD* 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 Δ*sigD* strains were performed from bacterial cultures growing for four weeks and optical density values at 600 nm (OD_{600}) were measured every 24 h. Cells were harvested at exponential (OD_{600}=0.4) and stationary (OD_{600}=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 anaerobic 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 µg/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 Δ*sigD* 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 *clp*X (as positive controls) and *lipY* and *rpf*B as (negative controls), according to Calamita *et al*., 2005. Specific gene primers (*Table 1*) were designed with the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) (Untergasser *et al*., 2007). For LightCycler reactions, a master mix of the following components was prepared: 3.0 µl of PCR-grade water, 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 Δ*sigD* 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 (McCarter 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.*

Gene (Name, CDC1551)*	*Sequence (5' to 3')*	*Product name*	*Reference*
ssd (MT3760)	F: CTGGGCGGCTGCCATAA R: CACCGCCGACCAATTCA	Septum site determining protein (ssd)	This study
*Sig*D (MT3523)	F: TCACCCGGATGAACGAATTG R: ACGACAACACGCAGAATGAG	Alternative sigma factor (SigD)	This study
*fix*A (MT3113)	F: TGATCAAGCAGGTCCCAGATAC R: TCTTTCTCCCGAATCTGTAGCG	Electron transfer flavoprotein (FixA)	This study
*lip*Y (MT3181)	F: GCAAGTGCCGATTTACCC R: CCACGCTGACGTTGGAGAC	Triacylglycerol lipase (esterase/lipase, LipY)	Soto-Ramírez *et al.*, 2017
*clp*X (MT2532)	F: TCTGATCAAGTTCGGCCTGATC R: TTGACCAAAGCGTTCTTCGG	CLP protease ATP-binding subunit (ClpX)	This study
*rpf*B (MT1038)	F: CCGCAATCGGATCAAGAA R: CGACCTCCCGGCTCAT	Resuscitation promoting factor (RpfB)	Soto-Ramírez *et al.*, 2017
rrs	F: GTAATCGCAGATCAGCAACG R: TTCGGGTGTTACCGACTTTC	16S Ribosomal RNA	This study

*Data taken from Tuberculist database: www.tuberculist.epfl.ch

server.molgenrug.nl/index.php/prokaryote-promoters). In order to find a possible consensus motif, the predicted promoter was compared with the consensus recognition sequences of the known Sig*D*-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 Δ*sigD* 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 FtsI (Slayden and Belisle, 2009). Additionally, we can

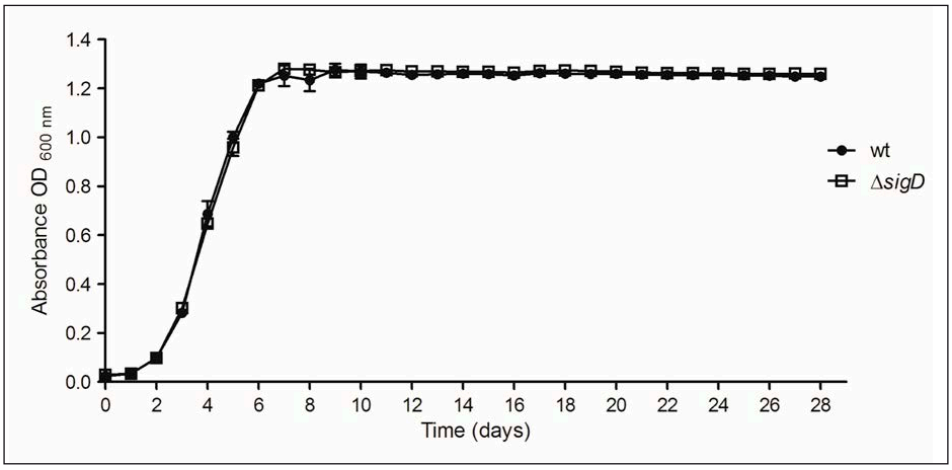

Figure 1 - *Growth curves of M. tuberculosis CDC1551 wild-type and ΔsigD mutant 347 strains at 37°C. Bacterial cultures were grown for four weeks in Middlebroock 7H9 348 medium supplemented with 10% ADC. OD values were obtained every 24 h.*

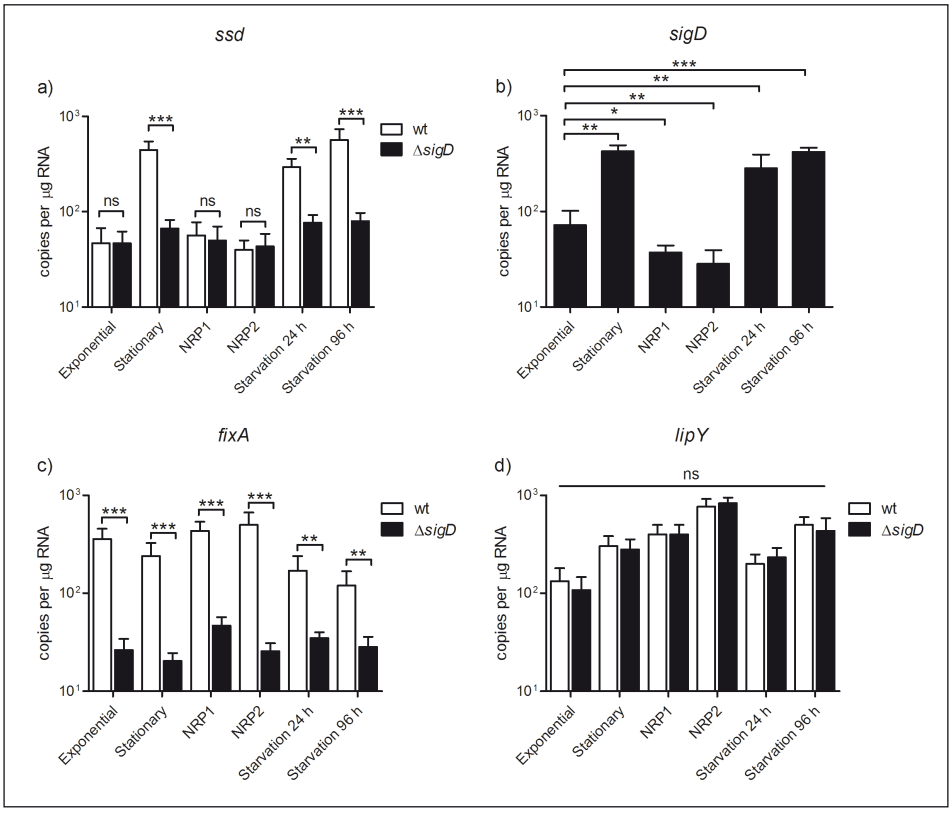

Figure 2 - *Expression of M. tuberculosis ssd, sigD, fixA and lipY genes during different stressful conditions. (a) Expresssion of ssd in the wild-type and ΔsigD mutant strains. (b) Expression of sigD in the wild-type strain. (c) Expression of fixA (positive link to sigD) in the wild-type and ΔsigD mutant 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.*

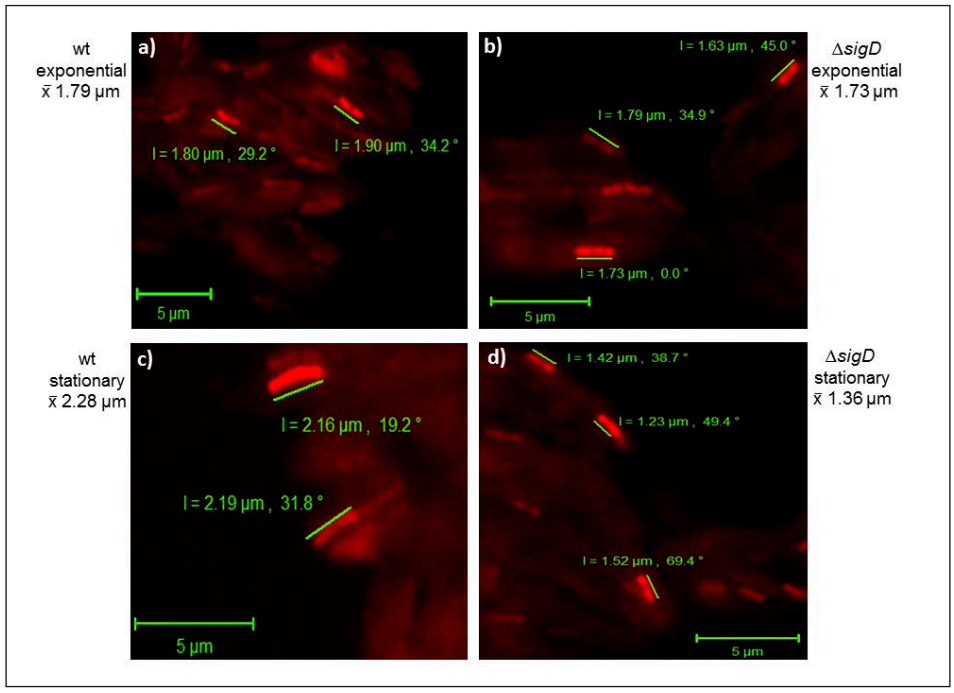

Figure 3 - *Putative role of* Sig*D on the cell length (elongation) of M. tuberculosis. Wild-type (a) and ΔsigD mutant (b) strains at exponential phase of growth. Wild-type (c) and ΔsigD mutant (d) strains at stationary phase of growth. The images were obtained by confocal laser scanning microscopy using a 100X oil-immersion objective for all magnifications. The average (x̄) of the bacilli length was calculated by observation of 20 fields in all cases. I, individual cell length of each bacillus.*

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 Sig*D* 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 Δ*sigD* mutant strain was similar to the wild-type (*Figure 2a*), we suggest that in the absence of Sig*D* some other regulators may exist which maintain incessant 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 Δ*sigD* 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 *Sig*D

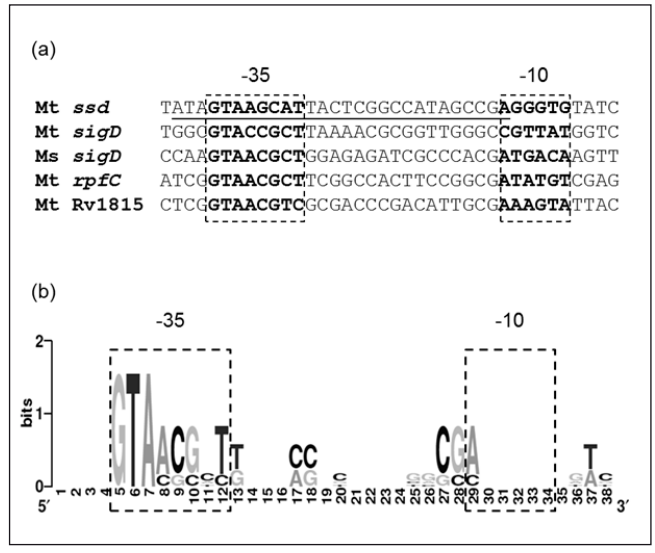

Figure 4 - *Alignment of the predicted promoter region from ssd gene of M. tuberculosis with the previously* Sig*D-dependent in vivo promoters determined by Raman et al. (2004). (a) The predicted promoter from ssd gene with the Genome2D web server is underlined. The -35 and -10 regions are in bold letters and framed with dotted line boxes. (b) Logo motif analysis with the WebLogo software of the predicted promoter regions. The -35 and -10 regions are indicated. M. tuberculosis (Mt); M. smegmatis (Ms).*

expression are linked to *ssd* expression under starvation conditions.

All gene expression results mentioned above were confirmed by a positive control gene (*fix*A) reported to be linked to *sig*D, 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 *clp*X gene, another positive control, was used (results not shown). In contrast, when a negative gene control (*lip*Y) 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 *lip*Y (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 Δ*SigD* cells stained cultures (harvested from exponential and stationary phases) with Auramine-Rodamine. We found that Δ*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, Sig*D* could produce a down-regulation of cell division and possibly an up-regulation of synthesis of the type IV pili 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.

Acknowledgments

*The authors would like to thank Dr T. Horn-Copeland, M.D., for the English language review of the paper. We also thank Dr William R. Bishai (from Johns Hopkins University School of Medicine, Baltimore, MD, USA) for the donation of the M. tuberculosis CDC1551 Δ*SigD *mutant strain to Dr Clara Espitia, and thank Rocío L. García-Reyes for her excellent technical assistance. Miguel A. Ares and Nora Rios-Sarabia were supported by a Grant from the IMSS, Mexico (FIS/IMSS/PROT/MD13/1252). Miguel A. Ares was supported by fellowships of BECADOS, IMSS (No. 2013-001); PRODESI, IMSS; and BEIFI, IPN.*

This work was supported by the International Cooperation UAM-Banco Santander and Latin America (Centro de Estudios de America Latina, CEAL), UAM, Spain. This work was also partially supported by grant CB-255181, CONACyT, México (SR-G).

Conflict of Interest

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

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