Discussion
**DISCUSSION**

*M. smegmatis* is a fast growing saprophytic mycobacterial species, widely used as a surrogate to understand *M. tuberculosis* gene function and virulence for a long time; but, very little is known about its own gene regulation machinery. Sigma factors, the primary determinant of gene regulation in bacteria, directly control transcription by providing promoter specificity to RNAP holoenzyme and help in transcription initiation. *M. smegmatis* genome encodes 28 putative sigma factors, out of which 25 belongs to group IV and one each to group I (SigA), II (SigB) and III (SigF). Detailed information about roles and regulations of *M. smegmatis* SigA (Gomez et al., 1998; Predich et al., 1995), SigB (Mukherjee and Chatterji, 2005), SigH (Fernandes et al., 1999) and SigE (Wu et al., 1997) are reported but no information about any other sigma factor is available.

SigF in *M. tuberculosis* is well studied and reported to control the expression of genes encoding for virulence factors and cell wall components (Geiman et al., 2004; Williams et al., 2007). When mycobacterial sigF was first isolated from *M. tuberculosis* using degenerate PCR, its orthologous genes were simultaneously identified in slow growing tuberculous mycobacterial species like, *M. bovis* and *M. avium*, but not in fast growing species like, *M. smegmatis* and *M. abscessus* (DeMaio et al., 1996). sigF in *M. bovis* BCG is expressed predominantly at stationary phase and the early expression of *M. tuberculosis* sigF was found to be incompatible with *M. bovis* BCG growth. It was argued that the presence of SigF during the exponential phase might be deleterious for *M. bovis* BCG, as it enabled the early expression of genes responsible for the onset of stationary phase (DeMaio et al., 1997). However, similar expression of *M. tuberculosis* SigF did not affect *M. smegmatis* growth and it was suggested that the SigF regulon is missing in *M. smegmatis* (DeMaio et al., 1997). Recent sequencing of *M. smegmatis* and other environmental mycobacterial genomes revealed that sigF is present in all mycobacterial genomes studied except *M. leprae* where it exists as a pseudogene (Table 3.1). Though SigF from different mycobacteria have high level of sequence similarities among them, they form two distinct clusters separating SigF from slow and fast growing mycobacteria (Fig. 3.4). The segregation profile suggests possible functional divergence of SigF in slow and fast growing mycobacteria (Singh and Singh, 2008).

In the present study we have investigated the role of *M. smegmatis* SigF. Using a recombinant *M. smegmatis* strain carrying promoter reporter fusion construct, we
analyzed the expression pattern of sigF under varying growth conditions. We further generated a ΔsigF mutant, studied its phenotype under different physiological conditions and performed DNA microarray hybridization in order to characterize SigF regulon.

4.1 Role of SigF in M. smegmatis Biology

Unlike M. bovis BCG where sigF is expressed predominantly in stationary phase (DeMaio et al., 1996), we observed continued expression of sigF in M. smegmatis throughout the growth of bacterium at a level comparable to sigA expression (Fig. 3.9A). It suggests that the SigF regulon in M. smegmatis comprises genes from each stages of the growth and/or genes that are probably constitutively transcribed. Corroborating to this in our transcriptome analyses we observed nearly equal number or genes being downregulated during log and stationary phase (Fig. 3.22). This might be the reason, why early expression of sigF in M. smegmatis did not affect the growth of bacterium, as reported in earlier studies by DeMaio et al. (1997).

Using usfX-lacZ reporter fusion construct, we observed increased sigF expression when M. smegmatis cells were exposed to isoniazid and ethambutol but not to rifampicin and streptomycin. Treatment with SDS and exposure to cold shock also induced sigF expression in M. smegmatis (Fig. 3.7B). RT-PCR data for sigF expression (Fig. 3.9B) duly supported the reporter based expression profile suggesting that the changes noticed in the β-gal level directly relates to the altered level of expression obtained through sigF upstream promoter element(s) (Fig. 3.7B). The fact that the sigF expression increases after these treatments suggests that its regulon could be involved in the survival of the bacterium under aforesaid conditions. But none of these stress conditions affected the survival of M. smegmatis ΔsigF mutant strain SFKO1 when compared to the wild type. Thus, while M. smegmatis sigF expression is induced upon these treatments, increased sigF expression does not appear to be required for bacterial survival during subjected stress conditions. This reflects the abundance of overlapping stress response regulatory networks in this environmental mycobacterial species. It may be noted that SigF belongs to the class of alternative sigma factors, which exhibit overlapping network of regulon.

Fernandes and co-workers had demonstrated the functional overlap of M. smegmatis SigE and SigH (Fernandes et al., 1999). Expression levels of sigE and sigH rise after heat shock but survival of M. smegmatis ΔsigH and ΔsigE at elevated temperature was found to be comparable to its wild type counterpart (Fernandes et al., 1999; Wu et al., 1997).
However, *M. smegmatis ΔsigE/ΔsigH* double mutant was more sensitive to the elevated temperature than wild type and either of the single mutants (Fernandes *et al.*, 1999).

Isoniazid and ethambutol specifically target cell wall biosynthesis process in mycobacteria, whereas SDS is an ionic detergent that affects the cell wall architecture. Overexpression of SigF in *M. tuberculosis* leads to the differential regulation of many cell wall associated genes, suggesting a regulatory role for SigF in structure and function of the mycobacterial cell wall (Williams *et al.*, 2007). To see whether SigF in *M. smegmatis* has any role in cell structure or not, we compared the cell wall ultrastructure of *M. smegmatis* wild type and ΔsigF mutant using transmission electron microscopy. Electron micrographs revealed an altered organization of outer most layer of cell envelope in ΔsigF mutant. Outer most layer of *M. smegmatis* is composed of glycopeptidolipids (GPLs). Ruthenium red strongly reacts with the surface GPLs of mycobacteria which appears as electron dense outer layer in electron micrograph (Etienne *et al.*, 2002; Rastogi and Barrow, 1994). We observed an uneven distribution of electron dense materials, on the periphery of SFKO1 cells in contrast to evenly distributed electron dense layer seen in isogenic wild type strain (Fig. 3.20). GPL structure is based on a tripeptide aminoalcohol N-linked to a long chain fatty acyl residue. The tripeptide aminoalcohol moiety is nonribosomally assembled by the products of the *mps* genes (Billman-Jacobe *et al.*, 1999). An *M. smegmatis mps* mutant was shown to lack the outer most electron dense layer completely and it was observed that absence of staining in outer layer of the mutant was due to the absence of GPLs from the surface of the mutant (Etienne *et al.*, 2002). In *M. smegmatis*, GPL biosynthesis involves approximately 30 genes, all clustered together at a locus encompassing 65 kb region (Ripoll *et al.*, 2007). We looked at the expression level of these genes in our microarray data but, did not find any noticeable change in the expression of genes present in GPL cluster. It is possible that the total GPL content remains unchanged but the transport and distribution of GPLs may be affected in ΔsigF mutant. In fact many genes encoding transport and binding proteins were found to be downregulated in SFKO1 in microarray experiment (Table 3.4); some of which may be involved in the process of transport and/or distribution of GPLs across the cell wall. A detailed qualitative and quantitative analysis of SFKO1 GPLs will help in elucidating the reason for the altered distribution of GPLs in mutant strain.
Mycobacterial cell wall is rich in unusual lipids and acts as barrier to many drugs. Alteration in surface architecture of *M. smegmatis* ΔsigF mutant did not affect the uptake of anti-mycobacterial drugs as susceptibilities of SFKO1 towards drugs like isoniazid, rifampicin, streptomycin and ethambutol remain comparable to that of wild type (Table 3.2). Similar observation was made for GPLs deficient *M. smegmatis mps* mutant, where MICs of several anti-mycobacterial drugs remained unaffected (Etienne *et al.*, 2002).

*M. smegmatis* mc²155 cells produce pale yellow carotenoid pigment. Deletion of sigF from *M. smegmatis* resulted in loss of pigmentation (Fig. 3.13). An ectopic expression of *M. smegmatis* sigF completely restored the pigmentation in SFKO1, suggesting that loss of pigmentation indeed is the consequence of sigF deletion and not a polar effect of mutation. Aromatic carotene isorenieratene is a characteristic pigment of almost all yellow-orange pigmented mycobacteria (Britton *et al.*, 1980). Metabolic pathway for isorenieratene synthesis uses farnesyl pyrophosphate as a precursor, which leads to isorenieratene in five metabolic steps involving, subsequently, CrtE, CrtB, CrtI, CrtY, and CrtU as illustrated in Fig. 3.14A. In *M. aurum* the biosynthesis of isorenieratene is directed by a carotenogenic gene cluster containing eight open reading frames, *crtEIBYcYdTUV*, each transcribed in the same direction (Viveiros *et al.*, 2000). Similar cluster of orthologous genes is present in *M. smegmatis* genome, containing the orthologs of six out of the eight open reading frames, involved in the biosynthesis of isorenieratene in *M. aurum* (Fig. 3.14B). *crtT*, encoding for methyltransferase, was shown to be dispensable for isorenieratene production in *Streptomyces griseus* (Krugel *et al.*, 1999) is missing and *crtE* gene, encoding the geranylgeranyl pyrophosphatase synthase, is not part of the locus and is found more than 1.8 Mb away (Fig. 3.14B). To check whether SigF directly regulates the expression of *crt* genes we analyzed upstream intergenic regions of *crtE* and *crtI* for the presence of SigF promoter signature using NNPP program and found a perfect SigF promoter signature upstream to the *crtI* (Fig. 3.14C). It was postulated that in the absence of SigF, transcription of *crt* operon is off, hence SFKO1 lacks pigmentation. This was further confirmed by qRT-PCR analyses of *crtI* transcript in SFKO1 where level of the transcript was found 20 fold less as compared to the wild type *M. smegmatis* (Fig. 3.15).

Presence of SigF consensus signature in the upstream region of *crt* operon (Fig. 3.14A) and repression of *crtI* in SFKO1 (Fig. 3.15 and Table 3.5) suggest that SigF directly
regulates carotenoid biosynthesis in _M. smegmatis_. _M. marinum_, another pigmented mycobacterial species, showed organization of _crt_ locus very similar to _M. aurum_ and _M. smegmatis_, and its upstream region, preceding _crtE_ gene (MMAR_4806), contains a promoter sequence "CATTAAagttatgaagcacatcGGGTAG" very similar to SigF dependent promoter. It suggests that SigF orthologs, like in _M. smegmatis_, likely regulate the expression of carotenoid biosynthesis genes (_crt_ cluster) in other pigmented mycobacteria as well. In fact carotenoid biosynthesis in _Streptomyces griseus_ and _S. setonii_, genus close to _Mycobacterium_, is also regulated by a sigma factor CrtS that belongs to the same family of stress response sigma factors as mycobacterial SigF and _B. subtilis_ SigB (Kato _et al._, 1995; Lee _et al._, 2001).

Oxidizing agents are toxic to cells. They generate reactive oxygen which can damage major cell components like lipids and nucleic acids. Cells employ several strategies to combat the toxic effect of oxidizing agents. In different bacterial species, cells have several oxidative stress response genes which are generally under transcriptional control of alternate sigma factors. Several alternative and ECF sigma factors have been shown to play a role in resistance of mycobacteria to oxidative stress. These include SigH (Manganelli _et al._, 2002; Raman _et al._, 2001) and SigJ (Hu _et al._, 2004) in _M. tuberculosis_ and SigH (Fernandes _et al._, 1999) in _M. smegmatis_. To investigate the role _M. smegmatis_ SigF in oxidative stress response, we treated SFKO1 cells with sublethal concentration of _H₂O₂_ and found that SFKO1 cells were more sensitive to the treatment as compared to the wild type cells. Susceptibility of SFKO1/sigF complemented strain towards _H₂O₂_ is comparable to the isogenic wild type strain suggesting that the increased sensitivity of SFKO1 towards _H₂O₂_ is indeed a consequence of _sigF_ deletion.

It may be noted that we did not observe any increase in _sigF_ transcript as well as protein levels after following _M. smegmatis_ cells' exposure to 10mM _H₂O₂_ (Fig. 3.19). This is in contrast to the oxidative stress response mediated by other sigma factors like SigH wherein expression of _sigH_ is increased after treatment of _M. smegmatis_ cells with oxidizing agents (Fernandes _et al._, 1999). We reasoned that the increased sensitivity of SFKO1 towards _H₂O₂_ may be because of diminished expression of oxidative stress response gene(s) which are under transcriptional control of SigF. Several genes are involved in peroxide generated oxidative stress response in mycobacteria like _oxyR, ahpC, furA, sodA, katG_ and _ideR_. While AhpC, SodA and KatG are directly involved in detoxification of cytotoxic effect of oxidative reagents, OxyR, FurA and IdeR are
regulatory proteins. In enteric bacteria, the oxidative stress response is mediated through the transcriptional regulator OxyR (Christman et al., 1985; Tartaglia et al., 1989) where OxyR coordinately regulate the expression of ahpC and katG. In most mycobacteria, with the exception of M. leprae, M. avium and M. marinum, the oxyR orthologous genes are inactivated by several mutations (Deretic et al., 1995; Pagan-Ramos et al., 1998; Sherman et al., 1995). Furthermore, ahpC and katG are controlled differentially in mycobacteria. Induction of an oxidative stress response must, therefore, be via a different mechanism in mycobacteria. Nevertheless, mutation in ahpC and katG both are implicated in increased susceptibility of mycobacteria to oxidizing agents (Pagan-Ramos et al., 1998). IdeR is an iron-responsive regulator and M. smegmatis ideR mutant was shown to have increased sensitivity to oxidative stress (Dussurget et al., 1996). This was shown to be due to reduced expression of catalase-peroxidase (KatG) and iron-dependent superoxide dismutase (SodA) (Dussurget et al., 1998). FurA is encoded by a gene immediately upstream of katG in all mycobacterial species tested, and was shown to be involved in the repression of katG in M. smegmatis (Zahrt and Deretic, 2001).

We did not observed any change in expression levels of genes encoding these proteins, suggesting that the increased sensitivity of SFKO1 towards H₂O₂ is not AhpC or KatG mediated. AhpC and KatG have been linked to the altered susceptibility of the bacterium to isoniazid (Dhandayuthapani et al., 1996; Heyrn et al., 1993; Zhang et al., 1992). Isoniazid is prodrug which requires cellular KatG activity to become active. Cell with non functional KatG are reported to me more resistant to isoniazid. The fact that susceptibility of SFKO1 to isoniazid is similar to the wild type (Table 3.2), further support the idea that increase sensitivity of SFKO1 to H₂O₂ is not KatG or AhpC mediated.

Carotenoids are a class of natural fat-soluble pigments found mainly in plants, algae, and photosynthetic bacteria, where they play a critical role in the photosynthetic process. They also occur in some non-photosynthetic bacteria, yeasts, and molds, where they carry out a protective function against damage by light and reactive oxygen. Loss of carotenoid pigmentation increases susceptibility of Rhodobacter spheroids to oxidizing agents like H₂O₂ (Anthony et al., 2005). In fact some of the nonpigmented mutants of M. marinum were shown to have similar resistance to methylene blue generated oxidative stress when compared to wild type cells. Increased susceptibility of ΔsigF mutant toward H₂O₂ could be attributed to the loss of carotenoid pigments. We are currently trying to
express *crt* operon under control of a constitutive promoter at ectopic locus in SFKO1 to address the role of carotenoid pigments in protection against oxidizing agents. However, we do not rule out the possibility of involvement of another pathway(s)/mechanism(s) for enhanced susceptibility of SFKO1 towards free peroxide radicals. In pigment less *R. spheroids* H$_2$O$_2$ elicits SigE mediated oxidative stress response (Campbell *et al.*, 2007). Whether nonpigmented SFKO1 cells employ an alternative defense strategy remains to be analyzed. Expression profiles of different sigma factors in SFKO1 after H$_2$O$_2$ treatment may provide the blueprint of sigma factor regulatory networks that assist *M. smegmatis* ΔsigF mutant against oxidative stress.

An *M. smegmatis* lsr2 mutant has been recently reported to be nonpigmented (Kocincova *et al.*, 2008) like ΔsigF mutant. Microarray analyses suggest that sigF expression is repressed in *M. smegmatis* lsr2 mutant (Colangeli *et al.*, 2007). No information about the susceptibility of *M. smegmatis* lsr2 mutant towards H$_2$O$_2$ is available; however, an *M. tuberculosis* lsr2 mutant was shown to be more sensitive to H$_2$O$_2$ treatment (Colangeli *et al.*, 2007). Response of *M. smegmatis* lsr2 mutant toward H$_2$O$_2$ too can provide an insight of carotenoid mediated protection against oxidative stress in *M. smegmatis* cells.

In a similar study Gebhard *et al.*, (2008) have shown that in *M. smegmatis* sigF expression does not increase after cold shock, isoniazid and ethambutol treatment. This could be because of the temperature used for cold shock was moderate 25 °C in comparison to 15 °C and concentrations of isoniazid and ethambutol used by them were far below than the concentrations used in the present study. They have shown that an *M. smegmatis* ΔsigF mutant similar to SFKO1 is more sensitive to H$_2$O$_2$ and results corroborate with our observation. However, they did not notice loss of pigmentation after sigF deletion. This could probably be because of the medium they used for cultivation of *M. smegmatis* strains. LB agar plates have yellowish colour and white *M. smegmatis* colonies on them look slightly yellow making it difficult to differentiate the mutant colonies from the wild type. Other possible reason could be the time of incubation. Yellow colour in wild type strains appears only after 10-12 days or longer incubation. In addition to this authors have shown that *M. smegmatis* ΔsigF mutant is more sensitive to acidic conditions and 52 °C heat shock. In our study, we did not observe any difference in survival of wild type and mutant strains at 42 °C (Fig. 3.17). Although we observed an overall decrease in viability of wild type and SFKO1 at 52 °C but survival of both strains was found to be by and large at similar level. Viability of both the wild type as well as
SFKO1 decreased approximately 50% at the given temperature. We did not study the effect of acidic pH on survival of SFKO1.

In another study with *M. smegmatis* ATCC607 strain, disruption of *sigF* resulted in loss of pigmentation and increased susceptibility to \( \text{H}_2\text{O}_2 \) (Provvedi *et al.*, 2008), which corroborated with our observations. However, in addition to these phenotypes, transformability of the *sigF* mutant 607 strain increased many fold (Provvedi *et al.*, 2008). *M. smegmatis* mc\(^2\)155 strain used in this study is a high transformation efficiency mutant originally isolated from its parent strain *M. smegmatis* ATCC607 (Snapper *et al.*, 1990). Deletion of *sigF* from *M. smegmatis* mc\(^2\)155 did not improve transformability of the strain further. It is interesting to note that *sigF* deletion in 607 strain increases its transformation efficiency, while mc\(^2\)155, a high transformation efficiency strain, contains functional *sigF* ORF, as demonstrated in our western analysis (Fig. 3.11). We argued that mc\(^2\)155 might be expressing lower level of *sigF* as compared to 607 and, therefore has enhanced transformation efficiency. Consequently overexpression of *sigF* from an extrachromosomal multicopy plasmid would revert mc\(^2\)155 strain back to low transformation efficiency phenotype. But overexpression of *sigF* did not bring in any change in transformation efficiency of mc\(^2\)155 (Table 3.3). We also compared the intrinsic level of *sigF* in both 607 and mc\(^2\)155 strains and found that level of *sigF* transcript and protein was comparable in both strains (Fig. 3.21). It is possible that high transformation efficiency of mc\(^2\)155 is the result of the defect in the expression of gene(s) not directly regulated by SigF in this strain. Also the differential post translational regulation of SigF cannot be ruled out as a possible reason for altered transformabilities of two *M. smegmatis* strains. It may be noted that *M. tuberculosis* CDC1551 \( \Delta \text{sigF} \) mutant had markedly reduced lag phase and grew to three fold higher density as compared to its isogenic wild type (Chen *et al.*, 2000), while a \( \Delta \text{sigF} \) mutant of *M. tuberculosis* H37Rv showed similar growth profile as compared to its wild type isogenic counterpart suggesting that the *sigF* may be differentially regulated in two strains of same species.

### 4.2 *M. smegmatis* SigF Regulon

Organization of *sigF* locus is conserved in mycobacteria wherein *usfX* gene, encoding for SigF cognate anti-sigma factor, precedes the *sigF* (Fig. 3.5). In all reported mycobacterial species but *M. avium*, *usfX* and *sigF* ORFs overlap suggesting the possible
cotranscription of both genes. In *M. tuberculosis* usfX and sigF were reported to be cotranscribed from a SigF dependent promoter situated upstream of the usfX (Beaucher et al., 2002; DeMaio et al., 1997). But in *M. smegmatis* sigF appears to be transcribed from two distinct promoters. One located in the upstream region of usfX while other was found in the upstream region of ChaB encoding gene MSMEG_1802 (Fig. 3.6A). MSMEG_1802 upstream promoter sequence matches to *M. tuberculosis* SigF consensus but sequence upstream to usfX does not resemble to *M. tuberculosis* SigF dependent promoter. Gebhard et al., (2008) identified two transcripts, one containing chaB-usfX-sigF and other comprising usfX-sigF. Using 5’ RACE (rapid amplification of cDNA ends) they identified two transcription start sites; an ‘A’ residue 17 bp upstream of the usfX start codon and a ‘G’ residue 41 bp upstream of the MSMEG_1802. Deletion of sigF resulted in decreased expression of chaB as well as usfX suggesting SigF autoregulate its expression, in part, if not completely.

Alternate sigma factors in mycobacteria show overlapping promoter specificities and are dependent on other sigma factors for their own transcription (Rodrique et al., 2006). Under standard physiological conditions and in response to SDS-mediated surface stress, sigB transcription is regulated by SigE (Manganelli et al., 2001), whereas in response to heat shock and oxidative stress sigB is transcriptionally induced by SigH (Manganelli et al., 2002). Similarly sigE is transcribed from multiple promoters in *M. smegmatis* and *M. bovis* (Wu et al., 1997); one of these resembles to SigH promoter consensus and SigH has been shown to regulate the sigE expression through this promoter upon exposure to heat shock and diamide (Raman et al., 2001) stress. SigF in *M. tuberculosis* is responsible for the expression of sigC; however, it does not appear to affect the transcription of any sigma factor gene in *M. smegmatis* as we did not find the down regulation of any sigma factor gene in SFKO1 in microarray experiments. Interestingly, we noticed the upregulation of two putative sigma factors encoding genes MSMEG_1418 (4.79 fold), and MSMEG_5365 (53.06 fold) in SFKO1 in log and stationary phase respectively. MSMEG_1418 encoded protein has 46% similarity to *M. tuberculosis* SigL while MSMEG_5365 has 58% similarity with *M. tuberculosis* SigL. SigF may negatively regulate these sigma factors by controlling the expression of their negative regulators. It is also possible that one or more sigma factors’ transcription is SigF dependent in changed physiological conditions as discussed above. Alternatively, it is also possible that SigF, SigL and SigI consensus sequences overlap, and in the absence
of SigF, other two sigma factors are increasingly synthesized to compensate the regulatory control missed by SigF in microbe.

*M. smegmatis* sigF is expressed throughout the growth of bacterium (Fig. 3.7A and 3.9A) and expectedly almost equal numbers of genes were found to be downregulated in log and stationary phase (Fig. 3.22). This is in contrast to the observation made in *M. tuberculosis* CDC1551 ΔsigF where more numbers of genes were found to be downregulated in stationary phase than log phase (Geiman *et al.*, 2004). This suggests that SigF in *M. smegmatis* is a constitutive gene and controls the expression of large number of genes from different stages of growth.

As sigma factors are positive regulator of transcription, genes regulated by SigF are likely to be downregulated in ΔsigF mutant and those under direct control of SigF would be downregulated during both log and stationary phases of growth. We analyzed primarily the downregulated genes and especially those commonly downregulated during both phases of the growth in SFKO1. It may be noted that while 309 genes were downregulated in log phase and 299 in stationary phase, the number of genes commonly downregulated during both phases were only 95. We speculate that these genes must be under the direct regulatory control of SigF as reasoned above. Many genes implicated in central intermediary and energy metabolisms were found to be repressed. Pathway analyses suggest that genes from carotenoid biosynthesis, sugar and nitrogen metabolism pathways were significantly downregulated in ΔsigF mutant. Carotenoid biosynthesis repression is apparent as SFKO1 colonies appear nonpigmented. Amino acid permeases and NarB (nitrate reductase) have role in nitrogen metabolism and genes encoding these proteins were highly repressed in SFKO1. The preferred nitrogen source for mycobacteria are amino acids, particularly asparagine, glutamate and aspartate, but they are able to use inorganic sources such as ammonium salts. NarB is assimilatory nitrate reductase and its orthologs are common in marine cyanobacteria where they reduce nitrate to nitrite and help bacterium to utilize nitrate as nitrogen source (Jenkins *et al.*, 2006; Moreno-Vivian *et al.*, 1999). Functional NarB may provide saprophytic *M. smegmatis*, an additional advantage to utilize nitrate as nitrogen source in nitrogen limiting conditions. Large number of energy and intermediary metabolism genes under control of an alternate sigma factor may ensure the survival of bacterium under changed nutritional situations. In fact under nutrient starvation condition sigF expression was found to be induced (Fig. 3.7B). Chowdhury *et al.* (2007) have demonstrated that SigF is
induced in sugar limiting growth conditions and controls expression of *dps* genes. *dps* genes are upregulated in starvation and oxidative stress conditions. *dps* promoter contains binding sites for both SigF and SigH. We did not observe loss of viability in SFKO1 under nutrient deprived condition.

Several transport and binding protein encoding and hypothetical genes were found to be downregulated in SFKO1; particularly, four hypothetical genes, MSMEG_6606, MSMEG_6607, MSMEG_6608, and MSMEG_6609, all clustered in genome as an operon. A BLAST search for MSMEG_6606 and MSMEG_6607 orthologs in protein data base resulted in identification of FtsK/SpoIIIIE proteins from *Frankia alni* and *Burkholderia pseudomallei* respectively. These proteins are involved in plasmid transfer/DNA segregation. Further analysis of this operon may provide some insight into the mechanism of the increased transformability of *M. smegmatis* mc²155.

Microarray studies were performed earlier for the characterization of *M. tuberculosis* SigF regulon (Geiman et al., 2004). Unlike SFKO1 where number of downregulated genes in log (309) and stationary phase (299) were almost equal, in *M. tuberculosis* ΔsigF mutant, more genes were downregulated in stationary phase (277 genes) than in log phase (36 genes) suggesting its predominant role in stationary phase (Geiman et al., 2004). In another microarray experiment where SigF regulon was characterized by over expression of SigF in *M. tuberculosis* (Williams et al., 2007). SigF regulated genes were found to be different from the ones observed by Geiman et al. (2004). They reasoned this discrepancy in observation to their different experimental approaches. In present study we considered data generated from both the experiments and compared with the list of genes downregulated in our study. We could not find any significant overlap in SigF regulons in *M. tuberculosis* and *M. smegmatis*. Williams et al. (2007) reported that large number of genes encoding cell wall associated proteins and PE/PPE were upregulated in *M. tuberculosis* as a result of SigF over expression. This includes of *mmpL* (mycobacterial membrane proteins large) family including *mmpL2, mmpL5* and *mmpL11* and PE/PPE genes Rv3136, Rv1441c, Rv1089 and Rv1195. No gene encoding *mmpL* family protein was found to be significantly downregulated in SFKO1. Same is the case with PE/PPE family protein encoding genes. Only few genes like *usfX, sigF* and gene encoding isocitrate lyase were found to be commonly downregulated in both mutants suggesting that there is very little overlap of SigF regulon between *M. smegmatis* and *M. tuberculosis*. It may be recalled that mycobacterial SigFs align to generate clusters that
separate slow and fast growing mycobacteria suggesting that, possibly, SigF addresses separate regulon in these species. Nevertheless, using tools like pattern search and NNPP, we identified SigF consensus signatures in *M. smegmatis*, NGTTTN-N$_{14-15}$GGGNAN which is similar to *M. tuberculosis* SigF. Like *M. tuberculosis* SigF consensus, ‘GGG’ in -10 hexamer is 100% conserved in *M. smegmatis* SigF cognate promoters.

We also analyzed the commonly upregulated genes during log and stationary phases of growth in SFKO1 mutant. It was argued that the biochemical deficiency created by diminishing level of proteins encoded by commonly downregulated genes in both phases must be compensated by transcriptional up regulation of their paralogs during same period of time. Consequently, commonly upregulated genes during both phases of growth must feature paralogs of commonly downregulated genes during these periods of growth. We identified 10 genes which are paralogs of genes downregulated during log and stationary phases of growth (Table 3.6). It may be noted that we have duly validated the expression level of several downregulated genes by real time RT-PCR analysis using gene specific primer pairs.

*M. tuberculosis* sigF gene (transferred along with its promoter) was induced in *M. bovis* BCG after exposure to various stresses; hypoxia, cold shock, oxidative stress, antibiotics like rifampicin, ethambutol, cycloserine, streptomycin, and entry into stationary phase (DeMaio *et al.*, 1996; Michele *et al.*, 1999). However, its induction was not observed in *M. tuberculosis* after cold shock, hypoxia, oxidative stress, or entry into stationary phase (Hu and Coates, 2001; Manganelli *et al.*, 1999). On the other hand *M. smegmatis* sigF was induced in response to cold shock, nutrient starvation, SDS, isoniazid and ethambutol. sigF and its upstream regulatory elements are 100% similar in *M. tuberculosis* and *M. bovis*, yet the regulation of its expression varied in these species. The nearly same consensus promoter elements are present in the *M. smegmatis* sigF upstream region, which exhibits entirely different sigF expression profile. These findings suggest that though the SigF regulon is marked with the conserved sequence signature in the promoter region, its binding to these regions is subject to the posttranslational regulation of these sigma factors, which are, in turn, regulated by changing cellular and external environments. These finding suggest that sigF activity may be regulated more strongly through its cognate anti-sigma and anti-anti-sigma factors instead of its transcriptional autoregulation (Beaucher *et al.*, 2002; Michele *et al.*, 1999).
The *M. tuberculosis* SigF is post-translationally regulated by its cognate anti-sigma factor UsfX, which is, in turn, regulated by two anti-anti-sigma factors, RsfA and RsfB (Beaucher et al., 2002). Both are able to disrupt the UsfX-SigF complex, releasing SigF to allow its association with RNA polymerase. The function of RsfA is regulated by redox potential, whereas the activity of RsfB is believed to be controlled by phosphorylation (Beaucher et al., 2002). We performed a BLAST search of the *M. smegmatis* genome and identified a protein (GenBank accession No. YP_890348) similar to RsfB. No RsfA-like protein was detected in the *M. smegmatis* genome. A search against other mycobacterial genomes showed the presence of an RsfA-like protein in tuberculous mycobacterial species. Among the environmental mycobacterial genomes, RsfA-like protein was detected only in *M. gilvum* (GenBank accession No.YP_001135681). Importantly, RsfA-like proteins are found to be present mainly in mycobacteria causing various form of tuberculosis. These findings suggest that SigF activity might be post translationally modulated by at least two distinct pathways in response to different physiological cues, the outcome being consistent with the bacteria's ability to adapt to diverse environments. The fact that RsfA has so far not been detected in *M. smegmatis* suggests that probably redox base regulation of the anti-anti-sigma factor is missing in this mycobacterial species. We need to study the SigF–anti-SigF interaction and its regulation by anti-anti-SigF in order to understand the mechanisms of sigF regulation in *M. smegmatis*. It might be noted that the *M. smegmatis* genome is much larger than *M. tuberculosis* and there are more than twice as many putative sigma factors in *M. smegmatis* as in *M. tuberculosis* (28 vs. 13) (Waagmeester et al., 2005). These findings suggest the presence of a larger network of genetic regulation in this species than in *M. tuberculosis* and it is possible that highly diverged regulatory circuits exist between these two mycobacterial species.

### 4.3 Conclusion and Future Prospective

We have shown that *M. smegmatis* sigF is a non essential gene as it is not required for growth of bacterium under normal growth conditions. Though, expression of sigF increases in response to cold shock, nutrient starvation and after treatment with antitycobacterial agents like isoniazid and ethambutol, its absence does not affect the survival of the bacterium under aforesaid conditions. Deletion of sigF resulted in loss of carotenoid pigmentation which probably increases the susceptibility of mutant towards \( \text{H}_2\text{O}_2 \) induced oxidative stress. SigF deletion also altered the structure of outer most layer
of the *M. smegmatis* cell envelope. *M. smegmatis* SigF controls the expression of variety of genes including several energy and central intermediary metabolism genes. Since *sigF* deletion does effect the growth of *M. smegmatis* but control pigment production and affect cell wall architecture, we consider SigF as a general stress response sigma factor with additional roles in pigmentation and in the maintenance of cell wall structure. We also derive the *M. smegmatis* SigF consensus signature NGTTTN-N_{14-15}-GGGNAN, which is very similar to *M. tuberculosis* SigF consensus. In spite of having highly similar consensus sequences, *M. smegmatis* and *M. tuberculosis* SigF regulons are different, which could be due to different distribution of these consensus sequences in the upstream regions of diversified genes.

It would be interesting to study the fate of other sigma factors expression in SFKO1, exposed to sublethal concentration of H$_2$O$_2$. This may provide some clue about how cell responds to oxidative stress in absence of carotenoid pigments. We would also like to investigate the underlying differences in transformation efficiency of *M. smegmatis* 607 ΔsigF mutant and mc$^2$155 strains. Low pigment level and increased transformation efficiency of mc$^2$155 could be because of differential post translational regulation of SigF. It requires to be further investigated.