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NOTES

SigF Controls Carotenoid Pigment Production and Affects Transformation Efficiency and Hydrogen Peroxide Sensitivity in *Mycobacterium smegmatis*▽†

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Carotenoids are complex lipids that are known for acting against photodynamic injury and free radicals. We demonstrate here that σ^F is required for carotenoid pigment production in *Mycobacterium smegmatis*. We further show that a *sigF* mutant exhibits a transformation efficiency 10^4-fold higher than that of the parental strain, suggesting that σ^F regulates the production of components affecting cell wall permeability. In addition, a *sigF* mutant showed an increased sensitivity to hydrogen peroxide. An in silico search of the *M. smegmatis* genome identified a number of SigF consensus sites, including sites upstream of the carotenoid synthesis locus, which explains its SigF regulation.

Although *Mycobacterium smegmatis* was originally isolated from humans, this fast-growing mycobacterium species is mostly nonpathogenic and has been used as a model to investigate mycobacterial physiology (1). When grown on agar plates, the wild-type strain ATCC 607 is characterized by a whitish morphotype, which turns to yellow-orange upon several days of exposure to visible light. Many mycobacteria produce yellow, orange or, less frequently, salmon pink pigments either in the dark (scotochromogens) or upon exposure to light (photochromogens). These pigments have been characterized as carotenoids, a class of polyterpene lipids that occur widely throughout nature and whose functions are to act as free radical scavengers and to protect the cells against photodynamic injuries (6, 13).

Transposon insertion into *sigF* determines loss of pigmentation. We constructed an *M. smegmatis* ATCC 607 insertional library using Tn*611* (10, 24). The mutants were visually screened for a pigmentation defect. Only 1 out of 15,000 library clones displayed a phenotype readily differing from that of the wild-type strain. Indeed, while the ATCC 607 wild-type strain formed yellow-orange colonies, the colony of the mutant strain had a whitish color (Fig. 1A). This mutant was characterized by using ligation-mediated PCR (21), which revealed an insertion in the open reading frame encoding the alternative sigma factor σ^F, which is homologous to stress response and sporulation sigma factors in many bacteria (22). The σ^F protein sequences of *M. smegmatis* and *Mycobacterium tuberculosis* are highly similar (29), and in *M. tuberculosis*, σ^F is a general stress factor that appears to play a regulatory role in the structure and function of the mycobacterial cell wall (30).

To prove that the loss of chromogenicity was specifically caused by the inactivation of *sigF*, we carried out complementation studies by expressing a wild-type copy of *sigF* at an ectopic locus within the mutant. In *M. smegmatis*, *sigF* seems to be part of the same transcription unit with *rsbW*, the gene encoding its putative anti-sigma factor, as in *M. tuberculosis* (3), since the *sigF* GTG start codon overlaps with the TGA stop codon of *rsbW*. Therefore, a fragment of 753 bp, containing the putative ribosome binding site upstream of the *rsbW* stop codon and the entire coding sequence of *sigF*, was amplified by PCR and cloned into the integrative expression vector pNIP40b between the XbaI and SpeI (indicated by lowercase letters) sites by using the primers RP283 (tctagaAAATTCCAGGAGGTCAGGTGACGTCGGAATACGCAGACGTTC) and RP271 (actagtGGCATTCCGAAGCGAGTTCC) (8, 28). The former primer was designed to contain an ribosome binding site (underlined). From previous experience, we know that cloning a gene at this site in pNIP40b leads to a transcriptional fusion with an upstream promoter and leads to expression of the transgene (7, 8, 28). The recombinant plasmid, pNIP40b/sigF, was introduced into the *sigF* mutant by electroporation as

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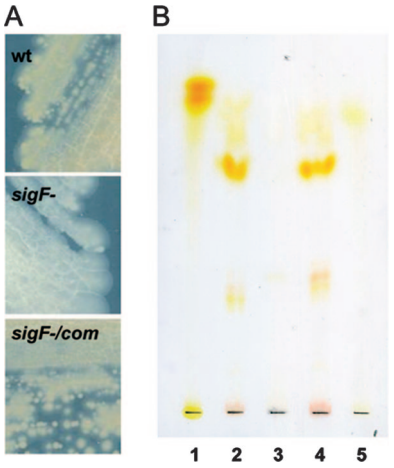

FIG. 1. A) Pigment production by the wild-type strain (wt), the *sigF* mutant, and the complemented strain. B) TLC of lipid extracts from the wild-type, the *sigF* mutant, and the complemented strains. TLC was run using $CHCl_3$-methanol (90:10), and no revelation has been performed. One mg of total lipid was loaded. Lanes: 1, β-carotene; 2, *M. smegmatis* ATCC 607 wild type; 3, *M. smegmatis* ATCC 607 *sigF* mutant; 4, *M. smegmatis* ATCC 607 *sigF* mutant (pNIP40B/sigF); 5, lycopene. The contrast of this image was improved by using processing software.

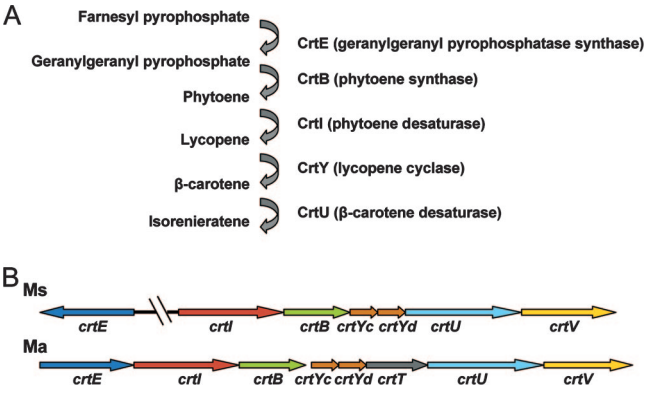

FIG. 2. A) Metabolic pathway leading to the production of isorenieratene in mycobacteria. Geranylgeranyl pyrophosphate (GGPP) is synthesized by CrtE (GGPP synthase) from farnesyl pyrophosphate. GGPP is converted to phytoene by CrtB (phytoene synthase). Phytoene is desaturated to lycopene by CrtI and transformed into β-carotene by CrtY (lycopene cyclase), an enzyme formed from the *crtYc* and *crtYd* gene products. The β-carotene is finally desaturated into isorenieratene by CrtU. B) Comparative genomic analysis of the carotenoid locus in *M. smegmatis* (Ms) and *M. aurum* (Ma).

previously described (20). After the pulse, the cells were diluted in 0.5 ml of liquid medium and incubated for 14 h at 37°C. The resulting recombinant strains were isolated on Middlebrook 7H10 solid medium containing 0.05% Tween 80 in the presence of both kanamycin (25 μg/ml) and hygromycin (50 μg/ml). Quantitative reverse transcription-PCR (RT-PCR) experiments performed with a *sigF*-specific primer (RP799, GTCGATGGACAGCGTGTTGT) to start reverse transcription demonstrated that the level of *sigF* expression in the complemented strain was similar to that exhibited by the wild-type parental strain (data not shown). Primers were designed to be able to detect transcripts containing the entire *sigF* gene and not those containing a truncated form present in mutant and complemented strains. For PCR amplification, primers RP481 (CTGGTCGGTGAAGGTGCC) and RP482 (GTACGAACT GCCCGCGAT) were used (the underlined base indicates the transposon insertion site in the *sigF* mutant). The recombinant strains were also examined for complementation of the nonpigmented phenotype. As shown in Fig. 1A, the introduction of *sigF* into the mutant fully restored pigment production. We also carried out complementation using the *sigF* gene from *M. tuberculosis*. Although less efficient, the *sigF* gene of *M. tuberculosis* was able to complement the pigment deficiency of an *M. smegmatis sigF* mutant, demonstrating that these genes are true orthologs (data not shown), as already suggested by in silico studies (29).

σ^F controls the expression of a gene cluster involved in carotenoid biosynthesis. Thin-layer chromatography (TLC) analysis of the carotenoid fraction extracted from both wild-type and *sigF* mutant cells grown on 7H11 medium revealed four pale yellow pigment spots with different R_f values in the wild-type extract (lane 2) that were all missing in the extract of the mutant (lane 3) (Fig. 1B). These four yellow pigments were all present in the *sigF*-complemented strain (lane 4) (Fig. 1B). The more-apolar pigment, with an R_f value of 0.45, similar to that of the lycopene used as a control (lane 5), was purified by preparative TLC. It displayed a UV absorption spectrum similar to that of mycobacterial isorenieratene (formerly designated leprotene), with a λ_{max} at 432, 457, and 483 nm (data not shown) (22). The other spots (including the major one) likely correspond to intermediary metabolites of the carotene family. The biosynthesis of the aromatic carotene isorenieratene is restricted to green photosynthetic bacteria and a few actinomycetes. Isorenieratene appears to be a characteristic pigment of almost all orange-pigmented mycobacteria (4), including *M. phlei* (12), *M. aurum* (18), *M. avium*, and *M. intracellulare* (26). A number of biochemical studies have documented the pathway leading to isorenieratene production (2, 16, 27). This metabolic pathway 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. 2A.

In *M. aurum* the biosynthesis of isorenieratene is directed by a carotenogenic gene cluster containing eight open reading frames, each transcribed in the same direction (27). Exploring the *M. smegmatis* genome, we found a gene cluster containing the orthologs of six out of the eight open reading frames involved in the biosynthesis of isorenieratene in *M. aurum* (*crtIBYcYdUV*). The only gene missing in *M. smegmatis* was *crtT*, encoding a methyltransferase shown to be dispensable for isorenieratene production in *Streptomyces griseus* (16). Surprisingly, the *crtE* gene, encoding the GGPP synthase, was not part of the locus and was found more than 1.8 Mb away (Fig. 2B).

To check whether σ^F is required for regulation of expression of the *crt* genes, we measured the mRNA levels of *crtI* and *crtE* in the *sigF* mutant, the wild-type parental strain, and the complemented strain by quantitative real-time RT-PCR, using *sigA* mRNA as an internal invariant control (19). RNA was extracted from cells grown to mid-exponential phase at 37°C with

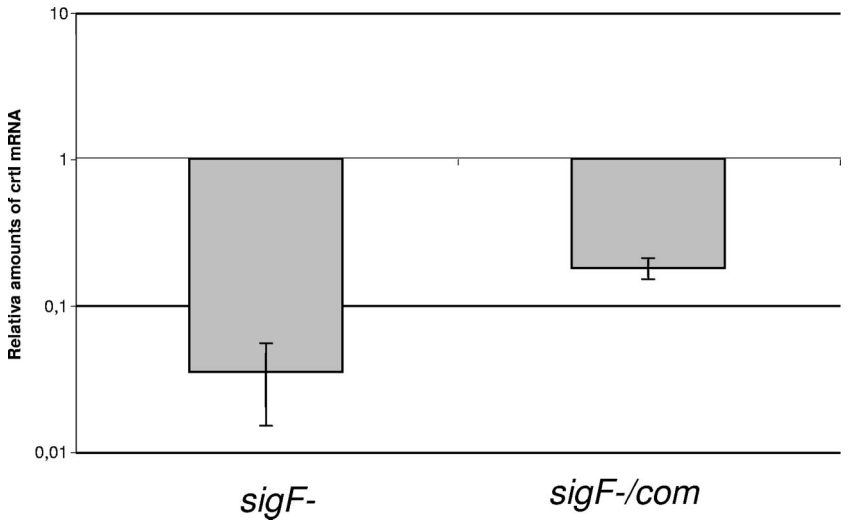

FIG. 3. Quantitative RT-PCR of the *crtI* gene in the *sigF* mutant and the complemented strain, showing relative amounts of mRNA with respect to the wild type.

shaking in Middlebrook 7H9 with 0.05% Tween 80. While *crtE* mRNA levels were the same in the three strains (data not shown), we found that the *crtI* mRNA level was reduced about 29-fold in the *sigF* mutant compared to the wild-type strain. This reduction was almost completely restored (80%) in the complemented strain (Fig. 3), suggesting that transcription of the *crtIBYcYdUV* cluster in *M. smegmatis* is under the transcriptional control of σ^F. In this regard, an *lsr2* mutant has been recently reported to be nonpigmented in *M. smegmatis* (15); a transcriptomic analysis has shown that the *sigF* gene is expressed at a low level in this mutant (5) and likely explains this lack of pigmentation.

Interestingly, carotenoid biosynthesis in *S. griseus* and *Streptomyces setonii* is also regulated by a sigma factor (CrtS) that belongs to the same family of stress response sigma factors as mycobacterial σ^F and *Bacillus subtilis* σ^B (14, 17), reinforcing the hypothesis that in *M. smegmatis* the *ctr* genes are under σ^F control.

Since the *M. tuberculosis* SigF consensus sequence is known (3), we searched the *crtI* upstream region for similar sequences. Interestingly, we found a perfect match at position bp −104 that suggested that the σ^F effect on *crt* gene transcription is direct and not mediated by another effector. To further support this hypothesis, we mapped the *crtI* transcription start site by 5′-rapid amplification of cDNA ends (Roche) and were able to show that it is indeed localized 9 bp downstream of the −10 region of the putative SigF-dependent promoter consensus sequence (Fig. 4).

Mutation of σ^F induces a dramatic susceptibility to hydrogen peroxide. As σ^F regulates the transcription of the *crtIBY cYdUV* gene cluster, which ultimately leads to a loss of pigmentation, and carotenoids are notable in their biology as free radical scavengers, we hypothesized that the *sigF* mutant might be hypersensitive to hydrogen peroxide. To this end we incubated the wild-type, *sigF* mutant, and complemented strains in 5 mM H_2O_2 (0.017%) and monitored cell death by CFU counting. After 2 h of incubation, the *sigF* mutant inoculum decreased more than 10^4 times, whereas the wild-type and the *sigF*-complemented strains remained unaffected by this free radical treatment (Fig. 5). This experiment clearly demonstrates that SigF, as in other mycobacterial species, regulates pathways that are important for stress survival and particularly toward oxidative stress. Interestingly, a recent study independently reported this finding in the mc^2155 strain and extended it to hypersensitivity to heat shock and acidic pH stresses (11). Whether these phenotypes are mediated in some way by carotenoid pigments remains to be tested. The pigment defect and hypertransformability were presumably not detected by Gebhard and colleagues, as the mc^2155 strain is less pigmented than the parental ATCC 607 strain (unpublished observation) and already hypertransformable (15).

The *sigF* mutant exhibits higher transformation efficiency

AAGACCGGACGTTTGTAGCCCGCCGCCTGCGGGTATCTC
GCGAG**C**ATGCCAAGCCCGGAAGGGGCGTATGTGAGCGAG
ATACCGACCTCGCCGGCGGTGTCCGCCGGCGCTTTCGTG
CTTTGCCGGGCGGCCCG**ATG**

FIG. 4. *crtI* upstream region. The −10 and −35 regions of the putative SigF-dependent promoter are underlined. The transcription start site and the translation start codon are shown in bold.

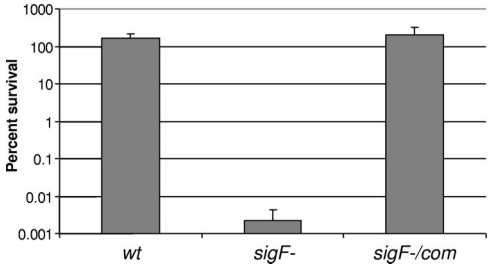

FIG. 5. Quantitative assessment of the bacterial survival of the wild-type, *sigF* mutant, and *sigF*-complemented strains following exposure to hydrogen peroxide.

than the parental strain. We noticed that the mutant appeared to be transformed with a higher efficiency than the parental strain. To check whether this difference was related to *sigF*, we characterized the wild-type, the *sigF* mutant, and the complemented strains for their abilities to be transformed by plasmid DNA. The replicative vector pLYG*Zeo* (9) was electroporated as described above, and transformants were selected with 100 μg/ml of zeocin (Invitrogen). The transformation efficiency of the wild-type strain was ≤10 transformants/μg of DNA, whereas that of the *sigF* mutant was $(9.5 \pm 1.2) \times 10^4$ transformants/μg (mean ± standard error). This efficiency was reduced more than 100 times when a functional copy of the *sigF* gene was reintroduced into the mutant ($[1.3 \pm 0.1] \times 10^2$ transformants/μg). These results strongly suggest the presence of genes in the SigF regulon that inhibit the uptake of DNA during electroporation. Interestingly, the common *M. smegmatis* reference strain mc^2155 is a mutant selected for its electroporation efficiency, which is 10^4 to 10^5 times greater than that of its parent strain (23). Whether or not this phenotype in mc^2155 is due to a defect in *sigF* or in one of its downstream genes awaits clarification.

In silico identification of the SigF targets in *M. smegmatis*. As the *M. tuberculosis sigF* complemented the *M. smegmatis* mutant and a perfect match with the *sigF* consensus sequence was found upstream of *crtI*, we probed the *M. smegmatis* genome for this sequence, using the string search tool Search Pattern (25). The word pattern search was GTTTC X_{14-18}GGGTAT (allowing two mismatches per hexamer) and limited to intergenic regions located between nucleotides −10 and −200 upstream of coding regions. A number of genes were shown to contain a putative *sigF* consensus in their upstream untranslated region, suggesting that they may be part of the *sigF* regulon. These genes belong to several functional classes, such as cell wall processes, intermediary metabolism, regulation, or unknown function. Interestingly, the orthologs of some genes that have been shown to be regulated by σ^F in *M. tuberculosis* (30) were also potentially *sigF* regulated in *M. smegmatis* (see Table S1 in the supplemental material). However, few genes appear to be *sigF* regulated in both *M. smegmatis* and *M. tuberculosis*, suggesting that the σ^F regulons have highly diverged between the two species.

Conclusions. Based on our data, we have shown that in *M. smegmatis* σ^F is involved in the regulation of genes required for carotenoid biosynthesis and probably other cell wall components. We also showed that the presence of a functional *sigF* gene decreases the *M. smegmatis* transformation efficiency, suggesting the involvement of some of the σ^F-regulated genes in the determination of cell wall permeability. The *sigF* gene is also involved in the resistance toward hydrogen peroxide. Finally, in silico analysis provided a list of potential σ^F targets in this species.

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