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Identification and expression of stressosomal proteins in *Mycobacterium marinum* under various growth and stress conditions

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Abstract

Like other bacteria, *Mycobacterium* spp. have developed different strategies in response to environmental changes such as nutrient limitations and other different stress situations. We have identified candidate genes (*rsb* genes) from *Mycobacterium marinum* involved in the regulation of the activity of the alternative sigma factor, σ^F. This is a homolog of the master regulator of general stress response, σ^B, and the sporulation-specific sigma factor, σ^F, in *Bacillus subtilis*. The organization of these genes in *M. marinum* and *B. subtilis* is similar. Transcriptome and qRT-PCR data show that these genes are indeed expressed in *M. marinum* and that the levels of expression vary with growth phase and exposure to stress. In particular, cold stress caused a significant rise in the expression of all identified *rsb* and *sigF* genes. We discuss these data in relation to what is currently known for other *Mycobacterium* spp.

Introduction

Species of the *Mycobacterium* genus are acid fast and robust; they inhabit various environmental reservoirs, for example, ground- and tapwater, soil, animals, and humans. The genus includes nonpathogenic environmental bacteria, opportunistic pathogens, and highly successful pathogens such as *Mycobacterium tuberculosis*, the causative agent of tuberculosis (Primm *et al.*, 2004; Vaerewijck *et al.*, 2005; Russell, 2007). Like other bacteria, *Mycobacterium* spp. compete for nutrients, and they have developed sophisticated ways to adapt to different environmental stresses. Here, both phosphorylation-dependent signal systems and alternative σ-factors play essential roles as switches that alter gene expression patterns globally (Storz & Hengge, 2011). A number of studies, of in particular *Bacillus subtilis* suggest that a specific protein complex, the stressosome (the 'RsbRST' complex), is involved in the activation of the alternative σ-factor, σ^B, in response to diverse environmental stresses (Pané-Farré *et al.*, 2005; Parida *et al.*, 2005; Hecker *et al.*, 2007; de Been *et al.*, 2011). Candidate genes encoding proteins that constitute the 'RsbRST' complex and that regulate the activity of σ^F, the σ-factor corresponding to the *B. subtilis* σ^B (and the sporulation σ-factor σ^F), have been identified in *Mycobacterium* spp. For example, *Mycobacterium avium* subsp. *paratuberculosis* (MAP) encodes homologs to the RsbRST module (*rsbR*, *rsbS,* and *rsbT*), while the σ^F anti-sigma factor, *usfX* (*rsbW*), and its antagonist, *rsfB* (*rsbV*), have been identified in *M. tuberculosis*. *M. tuberculosis* also encodes MursiF (*rv1364c*), a protein composed of domains that are also present in RsbV, RsbW, and RsbU. The gene encoding a homolog of the *B. subtilis rsbU* has not been identified in *Mycobacterium* spp. (Beaucher *et al.*, 2002; Pané-Farré *et al.*, 2005; Parida *et al.*, 2005; Sachdeva

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et al., 2008; see below). Information is scarce about when and under what conditions these genes are expressed and if the corresponding proteins mutually interact. Here, we have identified candidate stressosomal genes in the fish pathogen *Mycobacterium marinum*. Moreover, we present data showing that these genes are differentially expressed under different growth conditions and that the corresponding proteins interact in accordance with predictions based on studies of the *B. subtilis* system.

Materials and methods

Bacterial strains, media, and growth conditions

The *M. marinum* CCUG20998 attB::rfp-hyg^R strain was used. This strain contains the gene for RFP (red fluorescent protein) linked to the hyg^R gene and integrated into the genome at the L5 *attB* site (Mutoji & Ennis, 2012). Cells were grown at 30°C in 7H9 media supplemented with 100 μg mL^{-1} Hygromycin B. At $OD_{600\,nm}$ = 0.4–0.6 (exponential phase) or 3.6–4.6 (stationary phase), the cells were harvested by centrifugation (3820 ***g***) at room temperature. The pellets were frozen in liquid nitrogen and stored at −80°C. For heat and cold stress, the cultures were shifted to 42°C and 4°C, respectively, and incubated for an additional 24 h before harvesting, as described above (for the cold-stressed sample, centrifugation was performed at 4°C). Oxidative stress was induced by the addition of 10 mM H_2O_2 (Chauhan *et al.*, 2006; Wu *et al.*, 2007; Arnvig & Young, 2009) followed by further incubation for 24 h at 30°C. The stress-free control and anaerobic/microaerobic stress cultures were prepared by pelleting a sample of the exponential culture followed by addition of fresh prewarmed media. The former was incubated for another 24 h, while the latter was incubated without shaking and harvested after the disappearance of the blue color in a parallel culture treated with methylene blue (Chauhan *et al.*, 2006). $OD_{600\,nm}$ was measured at the time of harvest to monitor the effect of stress on cell growth (not shown).

RNA extraction, cDNA preparation, qRT-PCR, and RNA sequencing

Cells stored at −80°C were thawed on ice and resuspended in TRIzol (Invitrogen). RNA was extracted and converted to cDNA following the protocols given in supporting information. Quantitative RT-PCR (qRT-PCR) was performed with the primers listed in Table S1 and as described in supporting information, while RNA sequencing was performed at the SNP@SEQ Technology Platform at Uppsala University on a HiSeq2000 (Illumina) platform.

Cloning, bacterial two-hybrid system and assessment of positive protein–protein interactions

An *Escherichia coli* two-hybrid system (Karimova *et al.*, 1998; Ladant & Karimova, 2000; Euromedex) was used to study the interaction between different Rsb proteins. The different *rsb* genes were cloned pairwise into the plasmids pKT25 or pKNT25 and pUT18 or pUT18C as outlined in supporting information with the primers listed in Table S2. The resulting plasmids were transformed pairwise into the *E. coli* strain BTH101 (Str^R) and streaked on LA-plates supplemented with 50 μg mL^{-1} kanamycin, 100 μg ml^{-1} ampicillin, and 100 μg mL^{-1} streptomycin. Colonies were restreaked and plated on LA-plates containing 50 μg mL^{-1} kanamycin, 100 μg mL^{-1} ampicillin, 40 μg mL^{-1} X-Gal, and 0.5 mM IPTG according to the manufacturer's protocol; formation of blue colonies was scored as a positive result. Determination of β-galactosidase activity was carried out according to Miller (1972). As controls, β-galactosidase activities were measured using cells transformed with pairs of empty vectors. The significance of β-galactosidase activity data was analyzed statistically using the Student's *t*-test (two-tailed).

Antibodies and Western blot analysis

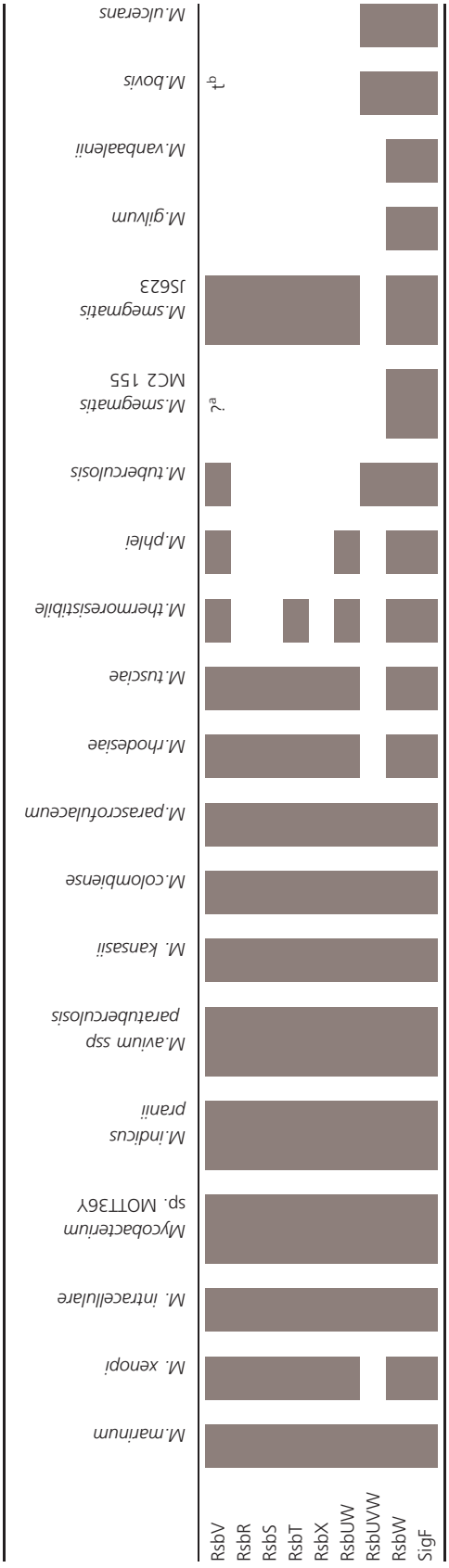
Genes encoding His-tagged *M. marinum* RsbR and RsbUW proteins were cloned (for primers, see Table S2) and expressed in *E. coli*; the proteins were purified and used to raise antibodies in rabbits (Innovagen). These antibodies were used to detect expression of RsbR and RsbUW by Western blot analysis following standard protocols in lysates of *M. marinum* cells that had been grown as described above.

Results

Candidate genes for a general stress pathway in *M. marinum*

To identify *M. marinum* genes involved in stress response, we used the M strain (Stinear *et al.*, 2008) and searched for homologs to known stress response genes in *B. subtilis*. The search was conducted as outlined in supporting information and Table 1. It appeared that all the genes (except *rsbU*) in the Rsb-mediated stress-signaling pathway are present in *M. marinum*, but the gene synteny is different compared with *B. subtilis* (Fig. 1). These genes are also present in the ATCC strain *M. marinum* CCUG 20998 (not shown). In the subsequent analysis, we have used the *B. subtilis* nomenclature, RsbV, RsbR, RsbS, RsbT, and RsbX, respectively. By analogy, MMAR_5186 is

Table 1. Presence of Rsb genes in *Mycobacterium* spp.



Filled box: gene present. Open box: gene absent. a: A gene exists that is annotated as *rsfB* which is an alternative name for *rsbV*; however, it is not very similar to the other mycobacterial RsbVs b: *M. bovis* has a truncated RsbV protein due to a point mutation disrupting the reading frame. Putative translation starts approximately one-third into the frame at a secondary site therefore its function must be questioned. The sequence data were obtained from NCBI ref seq protein data base using the BLASTP tool.

referred to as RsbUW due to the presence of domains homologous to both RsbU and RsbW. MMAR_3991 is referred to as RsbUVW rather than RsbU to reflect the sequence similarity with all the three proteins (RsbU, V, and W), while the RsbW annotation is used for MMAR_1247.

Gene expression profiles

To confirm that the genes were actually transcribed, we isolated total RNA from exponential and stationary cultures of *M. marinum* and performed transcriptome analysis by RNA sequencing (Fig. 2a). Transcripts from all *rsb* and *sigF* genes were detected, and the data suggest (after normalization) that the levels of the *rsb* and *sigF* gene transcripts were higher in exponential phase relative to those in stationary phase with the exception of *rsbV* and *rsbUVW*. However, the difference was not more than five-fold (*rsbR*). To confirm and validate these data, we determined the relative expression levels from at least two independent samples by quantitative real-time PCR (qRT-PCR). The cells were grown under the same conditions, as described for the transcriptome analysis. The overall patterns of expression were similar using the two methods (Fig. 2a) except for *rsbV*. On the basis of the transcriptome data, there appeared to be no significant difference in the level of *rsbV* in stationary and exponential phases. However, the qRT-PCR data suggest that it was significantly higher in exponential phase (P: 0.05). Moreover, the expression of *rsbR* in the two different phases did not display a significant difference between them. All other genes showed significantly higher mRNA levels in exponential phase (P: 0.05–0.01). On the basis of these data, it seems that the genes that are colocalized and share the same directionality (Fig. 1; *rsbR* to *rsbUW* and *rsbW-sigF*) have similar expression profiles in exponential and stationary phase grown cells. This is as to be expected if they are cotranscribed. By contrast, *rsbUVW* is transcribed at a significantly lower level, and this does not change to any significant extent when going from exponential to stationary phase. In conclusion, the *rsb* genes and *sigF* are expressed, and the expression levels vary with growth phase. In addition, the presence and expression of RsbR and RsbUW were confirmed by Western blot analysis using antibodies against these two proteins (Fig. 3).

Levels of *rsb* gene transcripts in response to stress

Next, we analyzed the levels of the various *rsb* and *sigF* transcripts in response to different stress conditions. *M. marinum* cells were grown in 7H9 media, and at

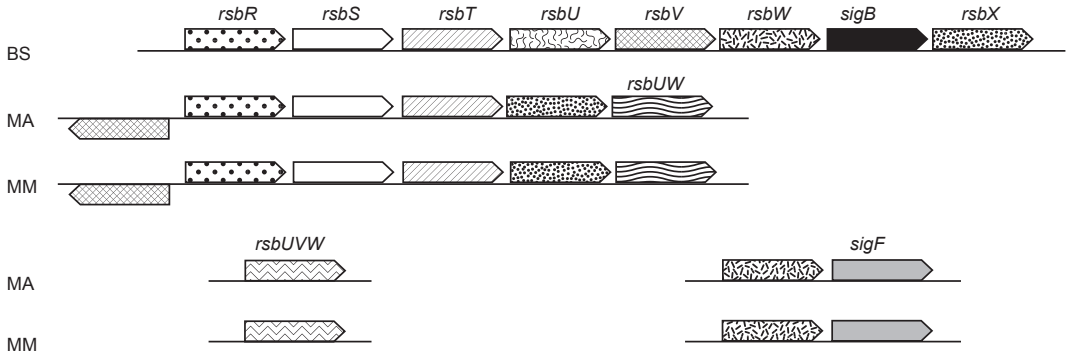


Fig. 1. Comparison of the genome organization of the *rsb* genes. The organization of the genes for the *B. subtilis* (BS) Rsb stress pathway proteins is shown together with putative genes of this pathway in *Mycobacterium avium* (MA) and *M. marinum* (MM) (see text for details). Boxes (not to scale) with identical and unique patterns indicate the corresponding genes and the name of the gene is defined for *B. subtilis* (or when absent in this organism, for *M. avium*). The pointed end of the boxes indicates the direction of transcription. Boxes aligned to the same solid line indicate that they are located next to each other in the same region of the chromosome.

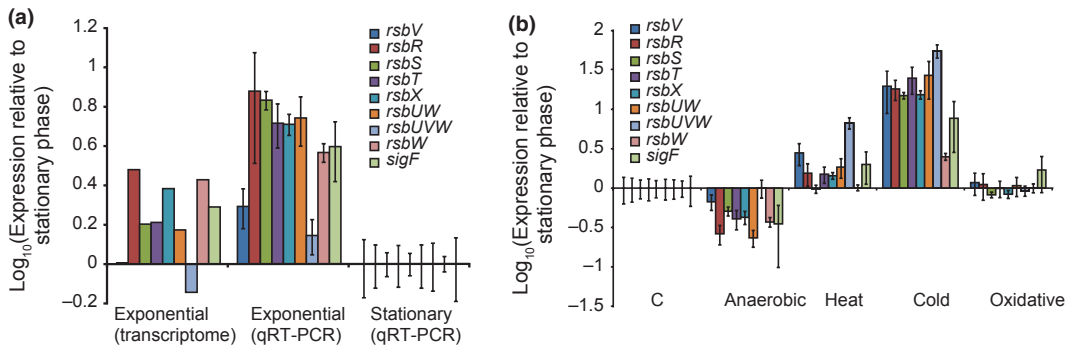


Fig. 2. Expression of the *rsb* genes during different growth and stress conditions. (a) The mRNA levels were quantified using RNA sequencing (transcriptome) or quantitative real-time PCR (qRT-PCR) from cells growing in exponential or stationary phase, and the ratio of exponential-to-stationary expression levels was calculated and plotted. The RNA sequencing data are based on a single replicate samples, while the qRT-PCR data show the average of at least two replicate samples with the experimental error indicated. Each gene is indicated by a different color. (b) *rsb* and *sigF* RNA levels were determined under various stress conditions by qRT-PCR. The average of at least two samples is shown with experimental errors indicated. The ratio for each stress condition in relation to the stress control (marked with a C) was calculated, and $\log_{10}$ values for these ratios were plotted. Anaerobic/microaerobic, heat, cold, and oxidative refers to the different stressors applied (see text for details). Bars below the *x*-axis indicate lower RNA levels than the stress control, and bars above the *x*-axis indicate higher RNA levels. Each gene is indicated by a separate color as defined in the figure. For qRT-PCR conditions, primers and probes see supplementary information and Table S1.

$OD_{600\,nm}$ = 0.4–0.6, the cultures were subjected in parallel to four different stressors: heat (42°C), cold (4°C), oxidative, and anaerobic/microaerobic for 24 h (Materials and methods). Total RNA was extracted, and the levels of *rsb* and *sigF* transcripts were determined by qRT-PCR. Figure 2b shows that the levels of all transcripts increased significantly when the cells were subjected to cold stress, while a modest increase was observed for the majority of the genes under heat stress conditions. No change was detected after the cells had been exposed to oxidative stress. Exposure to anaerobic conditions resulted in an overall reduction in the levels of both *rsb* (except *rsbV* and *rsbUVW*) and *sigF* transcription products. These data suggest that the levels of expression of these genes are subject to transcriptional regulation when *M. marinum* is exposed to certain stress conditions.

Rsb protein interactions

Considering the *rsb* gene synteny and that the various Rsb proteins are expressed in *M. marinum*, we decided to investigate whether the Rsb protein interactions are similar to those in *B. subtilis* (Pané-Farré *et al.*, 2005; Hecker *et al.*, 2007). For this purpose, we used a bacterial two-hybrid system as outlined in Materials and methods (Karimova *et al.*, 1998; Ladant & Karimova, 2000; see also

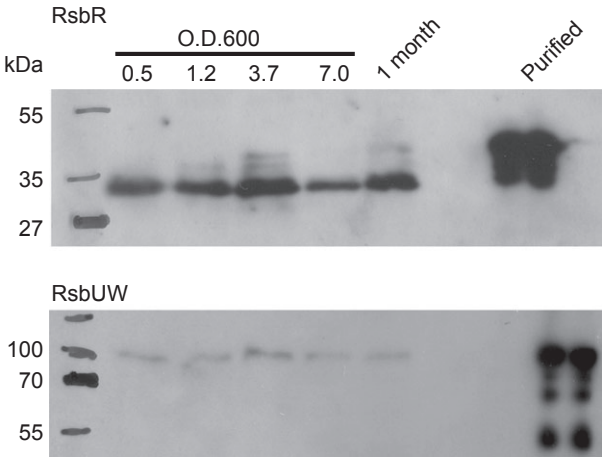


Fig. 3. Detection of RsbR and RsbUW. Western blot analysis using antibodies against RsbR and RsbUW as indicated. *M. marinum* cells were collected at different OD_{600} (0.5, 1.2, 3.7, and 7.0) and after 1 month of growth as described in Materials and methods. Purified His-tagged RsbR and RsbUW were used as controls. In particular, purified His-tagged RsbR showed a slower migration compared with native RsbR. Apart from the presence of the His-tag, the difference in migration might also be due to difference in the state of phosphorylation because the homologous proteins in *B. subtilis* are phosphorylated (Pané-Farré *et al.*, 2005; Hecker *et al.*, 2007; de Been *et al.*, 2011). The size markers were run at the same time, as the samples and their positions are marked with a pen.

Fig. 4). If two proteins interact, the resulting colonies are blue, due to the expression of β-galactosidase, on indicator plates supplemented with X-gal and IPTG (Fig. 4a). The β-galactosidase activity was subsequently quantified, and the results are presented in Fig. 4b. As summarized in Fig. 4c, these data suggest that RsbR interacts with RsbS and that RsbR interacts with itself. Likewise, RsbT interacts with itself as well as with RsbS, RsbUVW, and RsbUW. The latter two also interact with RsbV (the RsbW antagonist), which interacts with the anti-σ^F factor, RsbW. Although some of these interactions are weak, they are statistically significant (Fig. 4; Materials and methods) and consistent with the route (Pané-Farré *et al.*, 2005; Hecker *et al.*, 2007) leading to activation/deactivation of the alternative *B. subtilis* sigma factor, σ^B. For RsbX, we did not detect any interaction with any of the other Rsb proteins.

Discussion

In response to stress, bacteria activate alternative sigma factors. The RsbRST stress module plays an essential role in the σ^B-dependent general stress response in *B. subtilis* (Pané-Farré *et al.*, 2005; Hecker *et al.*, 2007; de Been *et al.*, 2011). The corresponding sigma factor in *Mycobacterium* spp. is σ^F, which also is a homolog to the sporulation-specific sigma factor σ^F in *B. subtilis* (DeMaio *et al.*, 1997; Sachdeva *et al.*, 2010). The RsbRST module is present in a number of bacteria including MAP (see introduction) and *M. marinum* (Pané-Farré *et al.*, 2005; this report), while this module has not been identified in *M. tuberculosis* and *M. smegmatis* MC^2 155 (Singh & Singh, 2008; Hümpel *et al.*, 2010; Table 1). In *B. subtilis,* the RsbRST is involved in controlling the activation of σ^B via the phosphatase RsbU, the anti-anti-sigma factor RsbV, and the anti-sigma factor RsbW (Pané-Farré *et al.*, 2005; Hecker *et al.*, 2007; de Been *et al.*, 2011). The activity of σ^F in *Mycobacterium* spp. is controlled in a similar way by UsfX, which corresponds to the *B. subtilis* anti-sigma factor RsbW (DeMaio *et al.*, 1997), and an RsbV homolog (Beaucher *et al.*, 2002; Parida *et al.*, 2005; Hümpel *et al.*, 2010; this report). No RsbU homolog has yet been identified in *Mycobacterium* spp.. However, two genes referred to as *rsbUW* and *rsbUVW* are present in, for example, *M. marinum* and MAP, while *M. tuberculosis* has *rsbUVW* (but not *rsbUW*). The homologous have RsbU, RsbV, and RsbW domains (RsbUVW) and RsbU and RsbW domains (RsbUW; Parida *et al.*, 2005). Therefore, RsbUVW and/or RsbUW may have a function in *Mycobacterium* spp. similar to that of RsbU in *B. subtilis* (see also Beaucher *et al.*, 2002; Parida *et al.*, 2005; Hümpel *et al.*, 2010). In addition, proteins that interact with either RsbW or σ^F or both have been identified in *M. tuberculosis* (Parida *et al.*, 2005; see also Hümpel *et al.*, 2010) further confusing the picture.

The genes encoding the *rsb* genes (except *rsbU*) and two additional genes, *rsbUVW* and *rsbUW*, are present in *M. marinum* and MAP. However, in *M. tuberculosis,* only *rsbV*, *rsbUVW* (also referred to as the MursiF gene), and *rsbW* have been identified (Table 1; see also, e.g. Parida *et al.*, 2005; Sachdeva *et al.*, 2008). Available *Mycobacterium* spp. genomes and data banks also reveal the presence of homologs to the *M. marinum rsb* genes in other species (Table 1). However, many *Mycobacterium* spp. lack several of these genes or perhaps the homologs have not yet been identified. For example, in *M. smegmatis* MC^2 155, no homologs have been identified, except *rsbW* (and *sigF*). However, Sachdeva *et al.* (2008) report that *M. smegmatis* MC^2 155 and others carry separate genes that encode domains similar to those present in RsbU, RsbV, and RsbW. Likewise, *Mycobacterium phlei* (Abdallah *et al.*, 2012; unpublished data) and *Mycobacterium thermoresistibile* lack most of the *rsb* genes. Thus, there seems to be no correlation between the presence or the absence of *rsb* genes and pathogenicity of *Mycobacterium* spp.. However, 16S rRNA phylogeny reveals that some of the species with a complete set of *rsb* genes cluster. The same is also true for those that lack at least one of the genes, but there are apparent exceptions, for example, *M. marinum* and *M. ulcerans* (see Fig.

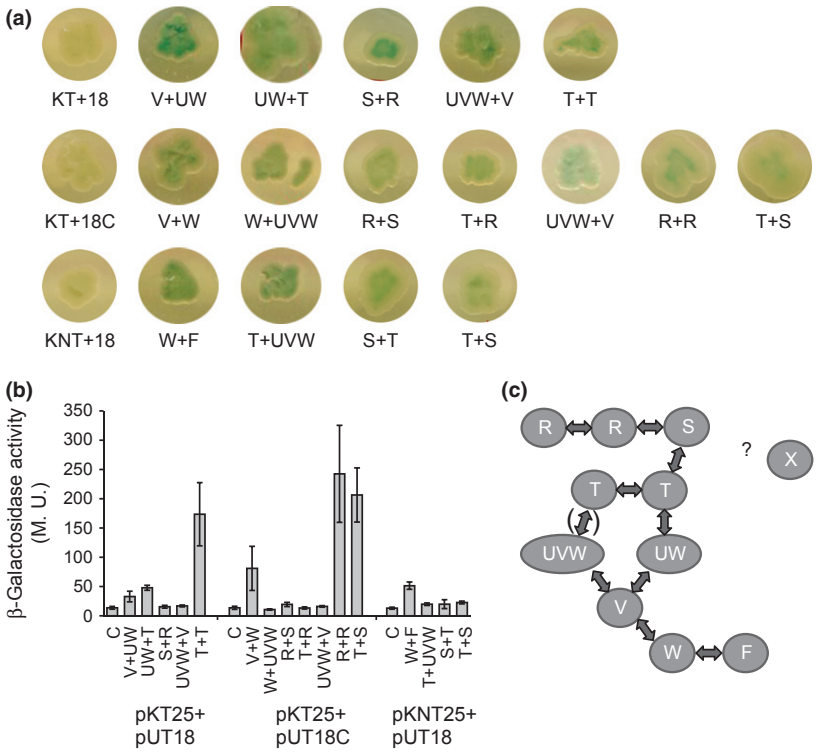


Fig. 4. Protein–protein interactions in the bacterial two-hybrid system. (a) *E. coli* BTH101 cells expressing various combinations of Rsb proteins or SigF were grown on LA-plates containing the chromogenic substrate X-Gal. Blue color indicates a positive protein–protein interaction. The top row shows cells transformed with the vector combination pKT25 and pUT18, the middle row shows cells transformed with the vector combination pKT25 and pUT18C, and the bottom row shows cells transformed with the vector combination pKNT25 and pUT18. The leftmost column shows cells transformed with the combination of empty control vectors indicated. KT, KNT, 18, and 18C represents pKT25, pKNT25, pUT18, and pUT18C, respectively. Letters before the plus sign were cloned in the pKT25 or pKNT25 vectors, and letters after the plus sign were cloned in the pUT18 or pUT18C vectors, as specified. R, S, T, UW, UVW, V, W, and X represents RsbR, RsbS, RsbT, RsbUW, RsbUVW, RsbV, RsbW, and RsbX, respectively, and F represents SigF. For a complete summary of all tested interactions, see Fig. S1. (b) Quantification of β-galactosidase activity for selected positive interactions. The bars are grouped according to the two-hybrid vector combination in which the genes were cloned. Their respective control (labeled C; cells transformed with the same combination of vectors without insert) specified below each group; these are the same (left to right) as in (a) (top to bottom). The order relative to the plus sign is as in (a). Each bar represents the average of at least three replicate samples with experimental errors as indicated. The β-galactosidase activity is expressed in Miller units (M. U.), and the bacterial two-hybrid system positive control (not shown) resulted in 110 Miller units. (c) Summary of all statistically significant interactions. Double-headed arrows indicate interactions and proteins are schematically represented by filled ovals. Letters represent Rsb proteins as defined in (a). A question mark at RsbX indicates that no interaction with any of the other proteins could be detected with any of the methods used, but that the question remains open (for details see the main text).

S2). Further studies are needed to understand the *sigF* activation pathway in those species where several of the *rsb* genes appear to be absent.

Our report shows that the *rsb* genes are present in *M. marinum* and expressed both in exponential and stationary growth phase. Moreover, on the basis of studies in *B. subtilis* and the *rsb* gene synteny in *Mycobacterium* spp., the various Rsb proteins are expected to interact. Our experiments using the two-hybrid system indeed suggested that these proteins interact with themselves and one another as predicted. Taken together with the data discussed above, we suggest that these proteins constitute the route involved in controlling the activation/deactivation of the alternative sigma factor σ^F, in response to environmental changes, at least in certain *Mycobacterium* spp. such as *M. marinum* but also in others that have the 'complete set' of these genes.

SigF and its anti-sigma factor RsbW (or *usfX*) are well conserved among *Mycobacterium* spp. (Sechi *et al.*, 2007; Singh & Singh, 2008). Previous studies have demonstrated induction of *sigF* in response to different environmental changes. For example, in *Mycobacterium bovis* BCG and *M. tuberculosis* σ^F induction of expression is triggered in response to various stress conditions including oxidative, stationary, and cold stress as well as oxygen depletion and exposure to antibiotics (DeMaio *et al.*, 1996, 1997;

Michele *et al.*, 1999). By contrast, heat stress results in a reduction of *sigF* expression in *M. tuberculosis* (Michele *et al.*, 1999), while no significant change was detected in *M. smegmatis* after exposure to heat for 4–8 h (Singh & Singh, 2008). Consistent with these data, we also found that transcription of *sigF* is affected when *M. marinum* is subjected to different stress conditions. However, the level and direction of change seems to vary dependent on species and type of stressor. This is also apparent when the expression of *sigF*, *rsbW*, *rsbV*, and *rsbUVW* in *M. tuberculosis* grown under different conditions is analyzed (Fig. S3). Hence, σ^F plays an important role in adapting to environmental changes, even in the aquatic living *M. marinum*. In addition, transcription of the *rsb* genes, which encode proteins involved in regulation of the σ^F activity, also changes in response to at least certain stress conditions. It will be interesting to find if σ^F itself or any other sigma factor has a role in the regulation of the *rsb* genes. Moreover, recent data suggest that the RsbRST route for activation of σ^B is not present in *Bacillus cereus*, *Bacillus anthracis* or several other *Firmicutes*. The activation route of σ^B in *Streptomyces coelicolor*, another *Actinobacteria*, represents yet another variation of regulation (de Been *et al.*, 2011). This raises the question whether these alternative regulatory pathways are used by some of the *Mycobacterium* spp. that lack the RsbRST module.

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References

Abdallah AM, Rashid M, Adroub SA, Arnoux M, Ali S, van Soolingen D, Bitter W & Pain A (2012) Complete genome sequence of *Mycobacterium phlei* type strain RIVM601174. *J Bacteriol* **194**: 3284–3285.

Arnvig KB & Young DB (2009) Identification of small RNAs in *Mycobacterium tuberculosis*. *Mol Microbiol* **73**: 397–408.

Beaucher J, Rodrigue S, Jacques PE, Smith I, Brzezinski R & Gaudreau L (2002) Novel *Mycobacterium tuberculosis* anti-σ factor antagonists control σ^F activity by distinct mechanisms. *Mol Microbiol* **45**: 1527–1540.

de Been M, Francke C, Siezen RJ & Abee T (2011) Novel σ^B regulation modules of Gram-positive bacteria involve the use of complex hybrid histidine kinases. *Microbiology* **157**: 3–12.

Chauhan A, Lofton H, Maloney E, Moore J, Fol M, Madiraju MV & Rajagopalan M (2006) Interference of Mycobacterium tuberculosis cell division by Rv2719c, a cell wall hydrolase. *Mol Microbiol* **62**: 132–147.

DeMaio J, Zhang Y, Young DB & Bishai WR (1996) A stationary-phase stress-response sigma factor from *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* **93**: 2970–2794.

DeMaio J, Zhang Y, Ko C & Bishai WR (1997) *Mycobacterium tuberculosis* sigF is part of a gene cluster with similarities to the *Bacillus subtilis* sigF and sigB operons. *Tuber Lung Dis* **78**: 3–12.

Hecker M, Pané-Farré J & Völker U (2007) SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu Rev Microbiol* **61**: 215–36.

Hümpel A, Gebhard S, Cook GM & Berney M (2010) The sigF regulon in *Mycobacterium smegmatis* reveals roles in adaptation to stationary phase, heat, and oxidative stress. *J Bacteriol* **192**: 2491–2502.

Karimova G, Pidoux J, Ullmann A & Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *P Natl Acad Sci USA* **95**: 5752–5756.

Ladant D & Karimova G (2000) Genetic systems for analyzing protein-protein interactions in bacteria. *Res Microbiol* **151**: 711–720.

Michele TM, Ko C & Bishai WR (1999) Exposure to antibiotics induces expression of the *Mycobacterium tuberculosis* sigF gene: implications for chemotherapy against mycobacterial persisters. *Antimicrob Agents Chemother* **43**: 218–225.

Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.

Mutoji KN & Ennis DG (2012) Expression of common fluorescent reporters may modulate virulence for *Mycobacterium marinum*: dramatic attenuation results from Gfp over-expression. *Comp Biochem Physiol C Toxicol Pharmacol* **155**: 39–48.

Pané-Farré J, Lewis RJ & Stülke J (2005) The RsbRST stress module in bacteria: a signalling system that may interact with different output modules. *J Mol Microbiol Biotechnol* **9**: 65–76.

Parida BK, Douglas T, Nino C & Dhandayuthapani S (2005) Interactions of anti-sigma factor antagonists of *Mycobacterium tuberculosis* in the yeast two-hybrid system. *Tuberculosis* **85**: 347–355.

Primm TP, Lucero CA & Falkinham JO 3rd (2004) Health impacts of environmental mycobacteria. *Clin Microbiol Rev* **17**: 98–106.

Russell DG (2007) Who puts the tubercle in tuberculosis? *Nat Rev Microbiol* **5**: 39–47.

Sachdeva P, Narayan A, Misra R, Brahmachari V & Singh Y (2008) Loss of kinase activity in *Mycobacterium tuberculosis* multidomain protein Rv1364c. *FEBS J* **275**: 6295–308.

Sachdeva P, Misra R, Tyagi AK & Singh Y (2010) The sigma factors of *Mycobacterium tuberculosis*: regulation of the regulators. *FEBS J* **277**: 605–626.

Sechi LA, Felis GE, Ahmed N, Paccagnini D, Usai D, Ortu S, Molicotti P & Zanetti S (2007) Genome and transcriptome scale portrait of sigma factors in *Mycobacterium avium* subsp. *paratuberculosis*. *Infect Genet Evol* **7**: 424–432.

Singh AK & Singh BN (2008) Conservation of sigma F in mycobacteria and its expression in *Mycobacterium smegmatis*. *Curr Microbiol* **56**: 574–580.

Stinear TP, Seemann T, Harrison PF *et al.* (2008) Insights from the complete genome sequence of *Mycobacterium marinum* on the evolution of *Mycobacterium tuberculosis*. *Genome Res* **18**: 729–741.

Storz G & Hengge R (2011) *Bacterial Stress Response*, 2nd edn. ASM Press, Washington, DC.

Vaerewijck MJ, Huys G, Palomino JC, Swings J & Portaels F (2005) Mycobacteria in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* **29**: 911–934.

Wu CW, Schmoller SK, Shin SJ & Talaat AM (2007) Defining the stressome of *Mycobacterium avium* subsp. *paratuberculosis in vitro* and in naturally infected cows. *J Bacteriol* **189**: 7877–7886.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Summary of all tested Rsb interactions in the bacterial 2-hybrid system.
Fig. S2. Phylogenetic analysis based on 16S rRNA.
Fig. S3. Comparison with *M. tuberculosis* gene expression data.
Appendix S1. Supplementary methods.
Table S1. Primers and probes for qRT-PCR analysis.
Table S2. Primers used for cloning, sequencing, and PCR.
Table S3. Summary of the blastp identification of the *rsb* and *sigF* genes.