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Differential regulation of the two-component regulatory system *senX3-regX3* in *Mycobacterium tuberculosis*

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The highly successful pathogen *Mycobacterium tuberculosis* (Mtb) has evolved strategies to adapt to various stress conditions, thus promoting survival within the infected host. The two-component regulatory system (2CRS) *senX3-regX3*, which has been implicated in the Mtb response to inorganic phosphate depletion, is believed to behave as an auto-regulatory bicistronic operon. Unlike other 2CRS, Mtb *senX3-regX3* features an intergenic region (IR) containing several mycobacterium interspersed repetitive units (MIRU) of unknown function. In this study, we used a *lacZ* reporter system to study the promoter activity of the 5′ untranslated region of *senX3*, and that of various numbers of MIRUs in the *senX3-regX3* IR, during axenic Mtb growth in nutrient-rich broth, and upon exposure to growth-restricting conditions. Activity of the *senX3* promoter was induced during phosphate depletion and nutrient starvation, and IR promoter activity under these conditions was directly proportional to the number of MIRUs present. Quantitative reverse transcriptase (qRT)-PCR analysis of exponentially growing Mtb revealed monocistronic transcription of *senX3* and *regX3*, and, to a lesser degree, bicistronic transcription of the operon. In addition, we observed primarily monocistronic upregulation of *regX3* during phosphate depletion of Mtb, which was confirmed by Northern analysis in wild-type Mtb and by RT-PCR in a *senX3*-disrupted mutant, while upregulation of *regX3* in nutrient-starved Mtb was chiefly bicistronic. Our findings of differential regulation of *senX3-regX3* highlight the potential regulatory role of MIRUs in the Mtb genome and provide insight into the regulatory mechanisms underlying Mtb adaptation to physiologically relevant conditions.

INTRODUCTION

One of the major obstacles to global tuberculosis eradication efforts is the unique ability of *Mycobacterium tuberculosis* (Mtb) to persist in the infected host within the inorganic phosphate (P_i)-poor phagolysosome of alveolar macrophages (Rengarajan *et al.*, 2005; Rifat *et al.*, 2009), or extracellularly within the necrotic debris of host lung granulomas (Grosset, 2003), which are depleted of nutrients (Gomez & McKinney, 2004) and oxygen (Haapanen *et al.*, 1959).

Mtb has evolved adaptive mechanisms, which promote survival within the infected host (Miller *et al.*, 1989). Bacterial adaptation to the environment is often controlled by a two-component regulatory system (2CRS) comprising a sensor histidine kinase (HK) and a response regulator (RR). Upon detection of an environmental stimulus, autophosphorylated HK transfers a phosphoryl group to the RR, leading to its activation, which enables DNA binding and initiation of gene transcription, resulting in the appropriate adaptive response (He *et al.*, 2006; Mayuri *et al.*, 2002). Eleven complete pairs of 2CRS and a few isolated HK and RR genes have been identified in the Mtb genome (Cole *et al.*, 1998), several of which have been shown to play an important role in the adaptive responses of the pathogen under stress conditions and in Mtb virulence (Converse *et al.*, 2009; Gonzalo Asensio *et al.*, 2006; Haydel *et al.*, 2012; He *et al.*, 2006; MacArthur *et al.*, 2011; Ohno *et al.*, 2003; Parish *et al.*, 2003).

Significant attention has focused on regulation of *senX3-regX3* and its role in Mtb virulence (Glover *et al.*, 2007; Himpens *et al.*, 2000; James *et al.*, 2012; Parish *et al.*, 2003; Rickman *et al.*, 2004; Rifat *et al.*, 2009; Supply *et al.*, 1997; Tischler *et al.*, 2013). In *Mycobacterium smegmatis*, SenX3-RegX3 has been shown to control expression of

Abbreviations: 2CRS, two-component regulatory system; C_2FDG, 5-acetylaminofluorescein di-β-D-galactopyranoside; HK, histidine kinase; IR, intergenic region; MIRU, mycobacterium interspersed repetitive unit; Mtb, *Mycobacterium tuberculosis*; P_i, inorganic phosphate; q, quantitative; RR, response regulator; RT reverse transcriptase; Tn, transposon.

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phosphate-dependent genes (Glover *et al.*, 2007), and is required for optimal growth under P_i-limiting conditions (James *et al.*, 2012). Mtb SenX3-RegX3 is also required for bacillary survival during P_i limitation (Rifat *et al.*, 2009), within THP-1 cells and activated murine macrophages (Parish *et al.*, 2003), and in mammalian lungs (Parish *et al.*, 2003; Rickman *et al.*, 2004; Rifat *et al.*, 2009).

Mycobacterial SenX3-RegX3, like other 2CRSs, has been described as an auto-regulatory operon (Glover *et al.*, 2007; Himpens *et al.*, 2000). Interestingly, the intergenic region (IR) between *senX3* and *regX3* consists of several mycobacterial interspersed repetitive units (MIRU) (Supply *et al.*, 1997). In other bacteria, such repetitive units may be involved in: (i) stabilizing upstream mRNA, thereby promoting differential gene expression or terminating transcription; (ii) controlling translation of downstream genes within a polycistronic operon; or, (iii) modulating host–pathogen interactions (Delihas, 2011; Newbury *et al.*, 1987). Although MIRUs have been used as target sequences for clinical diagnosis and as genotyping tools for epidemiological investigations (Magdalena *et al.*, 1998a; Millet *et al.*, 2007), their function remains to be determined in Mtb.

In this study, we used a *lacZ* reporter system to characterize the activity of the Mtb *senX3* promoter in response to P_i depletion and nutrient starvation. Using the same technique, we also studied the potential for various MIRU repeats within the *senX3-regX3* IR to independently regulate *regX3* expression under the same conditions. Using quantitative reverse transcriptase (qRT)-PCR, we investigated the transcription of the *senX3-regX3* 2CRS during exponential growth in 7H9 broth as well as the contribution of monocistronic and bicistronic transcription to Mtb *regX3* induction during phosphate depletion and nutrient starvation. Finally, we used Northern blotting and RT-PCR to confirm that upregulation of *regX3* in phosphate-depleted Mtb is chiefly due to increased monocistronic expression of the gene in Mtb CDC1551 and a *senX3*-deficient Mtb mutant (*senX3*::Tn).

METHODS

Growth conditions and bacterial strains. Supplemented Middlebrook 7H9 broth (Difco, BD), modified 7H9 broth containing 0 μM P_i (P_i depletion) (Rifat *et al.*, 2009) and 1× PBS (biological quality) containing 0.05 % Tween 80 (nutrient starvation) (Karakousis *et al.*, 2008) were used to study promoter activity and bacterial gene expression. P_i-depleted broth was prepared by reconstituting Middlebrook 7H9 broth (Difco, BD) except for the phosphate buffering components, which were replaced with 20 mM MOPS (pH 6.6), as described previously (Rifat *et al.*, 2009). Mtb CDC1551 (Ahmad *et al.*, 2010) was used as the wild-type strain in all experiments. A mutant deficient in MT0509/Rv0490/*senX3* was generated previously by mutagenesis of Mtb CDC1551 with the Himar1 transposon (Tn) (*senX3*::Tn; Tn insertion at bp 162/1233) (Lamichhane *et al.*, 2003).

Construction of promoter-containing recombinant strains. To study the promoter activities of *senX3* and the IR, the *Escherichia coli*–mycobacterium shuttle vector pYUB76 harbouring the *lacZ* gene (kind gift of Dr William R. Jacobs, Jr, Albert Einstein College of Medicine, NY, USA) was used (Barletta *et al.*, 1992; Glover *et al.*, 2007). Briefly, a 199 bp segment of the 5′ UTR of *senX3* was PCR-amplified using primers dl9-F and dl10-R (Table 1) from Mtb CDC1551 genomic DNA. A 254 bp PCR product, including the entire IR between *senX3* and *regX3*, as well as 16 bp of the 3′ *senX3* coding region and 40 bp of the 5′ *regX3* coding region, was amplified using primers IR-F and IR-R (Table 1). Following digestion with *Bam*HI (NEB), the *senX3* promoter and IR PCR products were individually ligated into *Bam*HI-digested pYUB76, yielding pSR and pIR, which were used separately to transform *E. coli* DH5α competent cells (Invitrogen). Clones were selected from LB agar plates containing kanamycin (50 μg ml^{-1}) and X-Gal (40 μl of 20 μg ml^{-1} stock). The identity of each clone was confirmed by DNA sequencing prior to electroporation of DNA constructs separately into wild-type CDC1551 competent cells and plating on kanamycin-containing 7H10 agar plates (Klinkenberg *et al.*, 2010).

Assessment of promoter activity by β-galactosidase assay. A standardized curve was generated by incubating β-galactosidase (0.0007 to 5 U ml^{-1}; Sigma) and 5-acetylaminofluorescein di-β-D-galactopyranoside (C_2FDG; Invitrogen) at a final concentration of 33 μM in 96-well plates (Cellstar) at 37 °C without exposure to light for 5 min, 30 min, 40 min and 72 h. A FLUOstar OPTIMA (BMG labtech) was used to read plates at excitation and emission wavelengths of 485 nm and 520 nm, respectively. Mycobacterial cell lysates were

Table 1. Primers used in this study

Primer	Sequence (5′→3′)	Purpose of amplification
dl9-F	cccggatccggtaattgtttgagatcccac	5′ UTR of *senX3*
dl10-R	cccggatcccagcgccgagaacacagtcac	
IR-F	cccggatccagagctgagccgatgacct	IR and co-expression
IR-R	cccggatccctcgtcctccacaatcaaca	
senX3-F	ccgagttgatcgagctatcc	*senX3* gene expression
senX3-R	agtgcggtaaccagcagagt	
regX3-F	tgttgattgtggaggacgag	*regX3* gene expression
regX3-R	cgcaactgcttgcatacatc	
sigA-F	ctacgctacgtggtggattc	*sigA* gene expression
sigA-R	ggtgatgtccatgtctttgg	
dl18-R	ccttgtcgatctcgctatcc	Biotinylated single strand *regX3* probe
SR-F	ccaaactctgctggttaccg	*senX3-regX3* co-transcript
SR-R	agcatcagatcgagcaggac	

sonicated (Thayil *et al.*, 2011) and the protein contents quantified for normalization purposes using a Qubit protein assay kit (Invitrogen). All samples were prepared in triplicate and incubated with C_2FDG for 72 h before measurement.

Gene expression analysis by qRT-PCR. Total RNA from wild-type CDC1551 was processed from nutrient- and P_i-starved cultures after 24 h and from mid-exponential phase cultures (OD_{600}=0.5) in Middlebrook 7H9 broth (Karakousis *et al.*, 2004; Thayil *et al.*, 2011). Gene expression levels were measured using the primer pairs listed in Table 1 and an iCycler 5.0 (Bio-Rad). cDNA synthesized with random hexamers (Invitrogen) was subjected to three technical replicates of PCR amplification, which were averaged (mean) to generate a single value for each biological replicate. The C_t obtained for each gene was normalized to that of the housekeeping gene *sigA* under each condition (Manganelli *et al.*, 1999). The amplification efficiency of primers targeting *senX3*, *regX3* and the bicistronic message (*senX3-regX3* co-transcript) was evaluated using CDC1551 genomic DNA, which was also used to calculate a ratio of fold per cycle for each primer set, with two independent experiments yielding similar results (data not shown). Statistical analysis was performed using three biological replicates for each sample.

Northern blot analysis. Northern analysis was performed using NorthernMax–Gly and BrightStar BioDetect kits (Ambion) according to the manufacturer's protocols. Briefly, total RNA was extracted from 72 h P_i-starved cultures of the wild-type strain, as described above, and separated on 1 % agarose gel, followed by transfer onto a positively charged nylon membrane (Ambion). A 274 bp single-stranded biotinylated *regX* probe was PCR-amplified using reverse primer dl18-R (Table 1) and purified by gel extraction prior to blot hybridization at 42 °C overnight. *regX3* mRNA was detected using streptavidin-alkaline phosphatase and CDP-Star (Ambion). The membrane was exposed to X-ray film for 1 h at room temperature and the film was developed in a dark room (AFP imaging).

Assessment of monocistronic and bicistronic expression of Mtb *senX3* and *regX3* by RT-PCR. Total RNA was extracted from 24 h P_i-starved cultures of wild-type CDC1551 and *senX3*::Tn. cDNA was synthesized using reverse transcriptase and oligo$(dT)_{20}$ primer as per the manufacturer's instructions (Invitrogen). cDNA corresponding to the *senX3-regX3* co-transcript was amplified using primers SR-F/SR-R (Table 1 and Fig. 5a). cDNA corresponding to *senX3* and *regX3* transcripts was amplified using primers *senX3*-F/*senX3*-R and regX3-F/regX3-R, respectively (Table 1 and Fig. 5a). Prior to amplification, the three primer pairs were tested using Mtb genomic DNA to confirm equivalent amplification efficiency. The amplified PCR products were run on a 1 % agarose gel containing ethidium bromide.

Statistical analysis. Means and standard deviations were calculated for each dataset. Differences between calculated means were compared by the Student's *t*-test. A *P* value $\leqslant$0.05 was considered statistically significant.

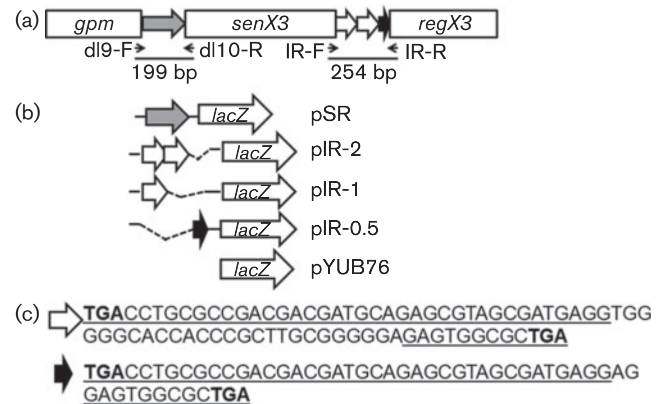

Fig. 1. Construction of promoter-containing Mtb recombinant strains. (a) Illustration of the Mtb *senX3-regX3* 2CRS and the strategy utilized to generate promoter-containing recombinant strains. The *senX3-regX3* IR consists of two identical 77 bp (white arrows) and a 53 bp (black arrow) MIRUs. (b) The 5′-UTR of *senX3* (shaded arrow) was cloned into the promoter-less plasmid pYUB76 upstream of the reporter gene *lacZ*, generating pSR. A similar cloning strategy was used for the *senX3-regX3* IR, yielding constructs pIR-2, pIR-1 and pIR-0.5 containing two full 77 bp MIRUs, one 77 bp MIRU and one 53 bp MIRU, respectively. (c) Nucleotide sequences are shown of the full 77 bp MIRU (white arrow) and the 53 bp MIRU (black arrow) within the *senX3-regX3* IR of wild-type CDC1551. Underlined sequences are common sequences shared by MIRUs and bold type indicates putative DTGA insertion sites.

RESULTS

The Mtb *senX3* promoter responds to P_i depletion and nutrient starvation

In order to determine if the Mtb *senX3–regX3* 2CRS behaves as a bicistronic operon in response to P_i depletion and nutrient starvation, we cloned the promoter region upstream of *senX3* from wild-type CDC1551 into vector pYUB76 (Table 2), which contains the reporter gene *lacZ* (Fig. 1a, b). Standard curves were generated to determine the sensitivity of the β-galactosidase assay using C_2FDG as a substrate (data not shown). The empty vector pYUB76 in Mtb showed a similar background under all experimental conditions (Fig. 2a, b). Relative to axenic growth in nutrient-rich broth, the *senX3*

Table 2. Plasmids used in this study

Name	Description	Source
pYUB76	*E. coli*–mycobacterium shuttle vector, promoter-less plasmid	William Jacob's lab, Albert Einstein College of Medicine, NY, USA
pSR	The 5′ UTR of *senX3* cloned into pYUB76	This study
pIR-0.5	A 53 bp MIRU cloned into pYUB76	This study
pIR-1	A 77 bp MIRU cloned into pYUB76	This study
pIR-2	Two 77 bp MIRUs cloned into pYUB76	This study

promoter responded to nutrient starvation and P_i depletion of Mtb ($P<0.05$) (Fig. 2a), consistent with a role for SenX-RegX3 in regulating the Mtb P_i starvation response (Rifat *et al.*, 2009). Expression of the *lacZ* reporter gene was strongest in nutrient-starved Mtb ($P<0.01$) (Fig. 2a).

The *senX3-regX3* IR responds to P_i depletion and nutrient starvation

The IR between *senX3* and *regX3* in wild-type Mtb CDC1551 comprises two identical 77 bp MIRUs and a 53 bp segment of MIRU containing common DTGA flanking sequences (Fig. 1a, c). In order to identify a potential regulatory function of the IR, we attempted to clone a 254 bp fragment comprising the entire IR and its flanking sequences into pYUB76 (Fig. 1a, b). Interestingly, various inserts were observed in a total of 20 sequenced clones purified from blue colonies on LB agar plates but none contained the intact region. Clones containing two intact 77 bp repeat units, a single 77 bp repeat or a 53 bp segment, flanked by 3′ *senX3* sequence and 5′ *regX3* sequence, were selected for further study, and designated pIR-2, pIR-1 and pIR-0.5, respectively (Fig. 1b and Table 2). Several other types of insertion or deletion products were also observed (data not shown).

Baseline promoter activity during Mtb growth in nutrient-rich broth was observed only for pIR-2 (Fig. 2b). Similarly, only pIR-2 showed promoter activity upon P_i depletion of Mtb, although promoter activity for this construct was significantly induced in P_i-starved Mtb relative to that observed in Mtb grown in nutrient-rich broth ($P<0.01$), suggesting that a minimum of two MIRU repeats is required for P_i-dependent expression of this promoter. Interestingly, although pIR-0.5 showed promoter activity in nutrient-starved Mtb, enhanced promoter activity of the IR in response to 24 h of nutrient starvation was observed with increasing number of repeats (Fig. 2b). As in the case of the *senX3* promoter, nutrient starvation was a more potent trigger of pIR-2 activity than was P_i depletion ($P<0.01$; Fig. 2b). Although cloning of the entire IR was not successful, the strong promoter activity observed in the construct containing two tandem MIRUs suggests equivalent or greater promoter activity for the intact IR.

senX3 and *regX3* are co-expressed but also differentially transcribed during nutrient-rich and stress conditions

In order to further identify a potential regulatory role for the *senX3-regX3* IR, we used qRT-PCR to study the abundance of *senX3*, *regX3* and *senX3-regX3* co-transcripts in wild-type CDC1551 during axenic growth in nutrient-rich broth, and following 24 h of P_i depletion or nutrient starvation (primer sequences listed in Table 1). Since the forward and reverse IR primers target the 3′ end of *senX3* and the 5′ end of *regX3*, respectively, detection of the amplified product indicates co-transcription of the operon. The three different primer pairs chosen to study *senX3*, *regX3* and *senX3-regX3* co-expression generated similarly sized PCR products with equivalent efficiency of amplification using the same concentration of DNA template (data not shown). If *regX3* expression is entirely bicistronic, i.e. dependent on the *senX3* promoter, the abundance of *regX3* transcripts would be expected to be equivalent to that of *senX3-regX3* co-transcripts. However, relative to *sigA* in nutrient-rich broth, the abundance of *senX3-regX3* co-transcripts was significantly lower than that of total *senX3* transcripts ($P<0.01$) or total *regX3* transcripts ($P<0.01$) (Fig. 3a), suggesting that *regX3* expression not only represents co-transcription with *senX3* but also includes monocistronic expression of *regX3*.

In nutrient-starved Mtb, expression of *senX3* and *regX3* increased to a similar degree as did that of *senX3-regX3*

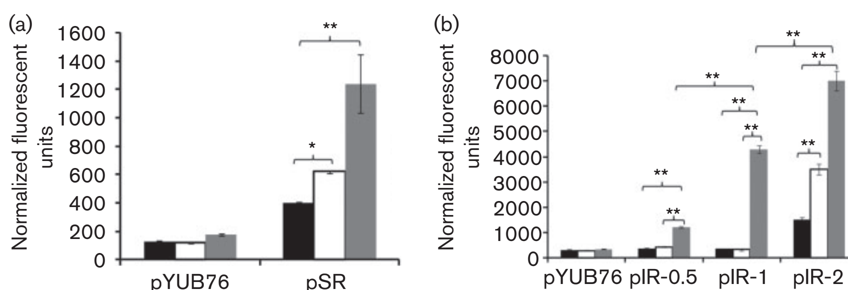

Fig. 2. Promoter activity of the Mtb *senX3* promoter and the Mtb *senX3-regX3* IR, as measured by β-galactosidase assay. Fluorescence units represent β-galactosidase activity produced by LacZ, whose expression is driven by the promoters of interest, after 24 h exposure to Middlebrook 7H9 broth (black bars), P_i depletion (0 µM P_i; white bars) and nutrient starvation (NS; grey bars). C_2FDG was used as fluorescent substrate. pYUB76-containing Mtb CDC1551 showed similar background activity, regardless of condition. (a) pSR showed stronger promoter activity following Mtb exposure to NS ($P<0.01$) and 0 µM P_i ($P<0.05$) relative to exponential growth in 7H9 broth. (b) Promoter activity of the IR increased with increasing number of MIRUs during NS ($P<0.01$). pIR-2 showed stronger promoter activity during Mtb exposure to NS ($P<0.01$) and 0 µM P_i ($P<0.01$) than during growth in 7H9 broth. Error bars represent SD of the mean. *$P<0.05$; **$P<0.01$.

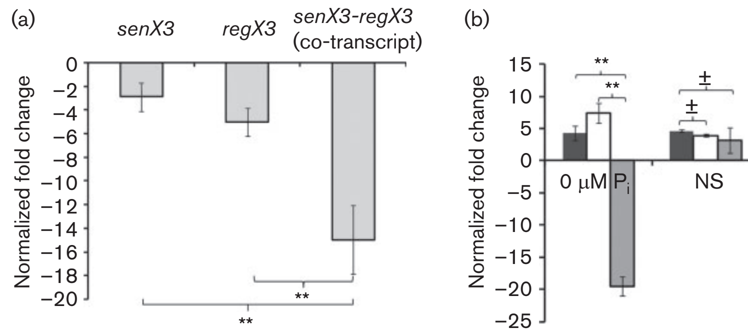

Fig. 3. qRT-PCR reveals that bicistronic and monocistronic expression of Mtb *senX3* and *regX3* is condition-dependent. Total RNA was purified from wild-type CDC1551 grown in Middlebrook 7H9 broth to mid-exponential phase, and under nutrient starvation (NS) and phosphate depletion (0 μM P_i) conditions for 24 h. Abundance of transcripts of *senX3* and *regX3*, and of *senX3-regX3* co-transcripts was calculated relative to that of *sigA*. (a) Abundance of *senX3-regX3* co-transcripts was significantly lower relative to that of *senX3* ($P<0.01$) and *regX3* ($P<0.01$) during growth in 7H9 broth. (b) Expression of *senX3* (black bars) and *regX3* (white bars) was upregulated, while that of the *senX3-regX3* co-transcript (grey bars) was downregulated, during Mtb exposure to 0 μM P_i relative to 7H9 broth ($P<0.01$ for both comparisons). By contrast, expression of *senX3*, *regX3* and the *senX3-regX3* co-transcript was upregulated to a similar extent during Mtb exposure to NS ($P>0.05$ for both comparisons). The level of expression of each gene was normalized to that of the housekeeping gene *sigA* under each condition prior to comparison between individual stress conditions and 7H9 broth. Positive values in these graphs represent increased gene expression and negative values represent decreased expression under each stress condition relative to 7H9 broth. Samples were prepared in triplicate under each experiment condition. Error bars represent SD of the mean. ±, $P>0.05$; $^{**}P<0.01$.

co-transcripts relative to nutrient-rich broth ($P>0.05$; Fig. 3b), suggesting that induction of Mtb *regX3* during nutrient starvation is primarily attributable to increased bicistronic expression (co-transcription of both genes). Interestingly, the transcriptional profile of these genes was different during P_i depletion relative to that during exponential growth in 7H9 broth. Thus, the level of *senX3* and *regX3* expression increased but expression of *senX3-regX3* co-transcripts dramatically decreased ($P<0.01$; Fig. 3b), suggesting that upregulation of *senX3* and *regX3* during P_i depletion is most likely monocistronic and driven by independent promoters. Northern blot analysis using biotinylated single-strand *regX3* probe (247 bp) revealed a ~800 bp band in the wild-type strain following 72 h of P_i depletion, confirming predominantly monocistronic transcription of *regX3* mRNA (Fig. 4). With longer exposure time, a very weak ~2.25 kb band representing the *senX3-regX3* co-transcript was observed (data not shown).

To further confirm the finding of monocistronic expression of *regX3* in P_i-depleted Mtb, we used RT-PCR to study the abundance of *regX3* and *senX3* transcripts in a *senX3*-disrupted mutant (*senX3*::Tn) compared with the isogenic wild-type strain following 24 h of P_i depletion (Fig. 5a). Using primer sets of similar efficiency (data not shown), we detected a very faint band of 795 bp in length, representing the *senX3-regX3* co-transcript, as well as two strong bands of 196 bp (*senX3*) and 192 bp (*regX3*) in P_i-depleted cultures of wild-type Mtb (Fig. 5b), demonstrating strong monocistronic expression of both *senX3* and *regX3*, and comparatively low levels of *senX3-regX3* co-transcription. Weak bands corresponding to *senX3* and *senX3-regX3* co-transcripts were observed in *senX3*::Tn, suggesting minor levels of read-through expression from the Tn insertion

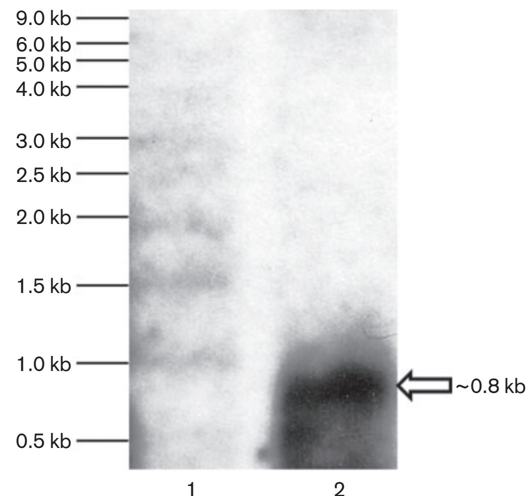

Fig. 4. Northern analysis confirms predominantly independent expression of Mtb *regX3* during P_i depletion. Biotinylated single-strand *regX3* probe (247 bp) was used for hybridization. Lane 1, BrightStar biotinylated RNA millennium marker (Ambion). Lane 2, total Mtb RNA following 72 h of P_i depletion. White arrow indicates a band corresponding to the independently expressed *regX3* transcript (~0.8 kb). The experiment was repeated using two biological samples with the same results.

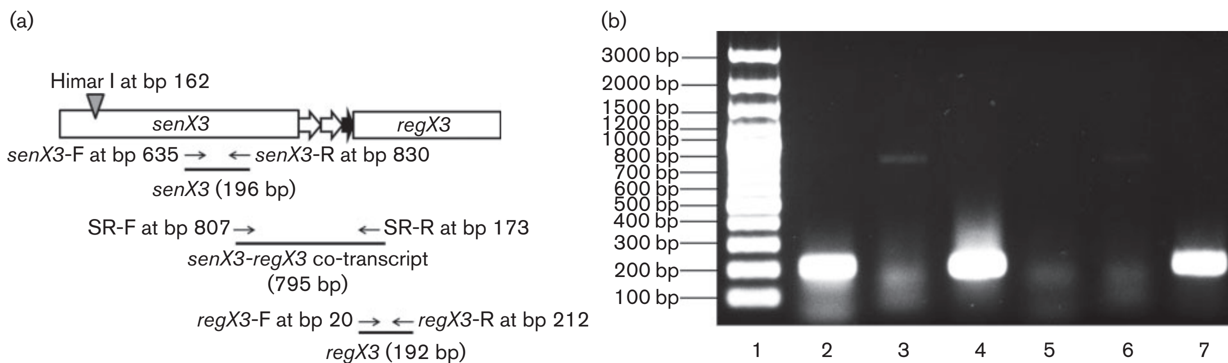

Fig. 5. Preserved expression of *regX3* by RT-PCR in a *senX3*-disrupted Mtb mutant strain during P_i depletion. Total RNA from 24 h P_i-depleted cultures of a mutant strain containing a Tn insertion in *senX3* (*senX3*::Tn) and the isogenic wild-type was used for cDNA synthesis with oligo(dT)$_{20}$ primer followed by PCR amplification. (a) Illustration of primer pairs used to amplify cDNA corresponding to the *senX3* transcript (senX3-F/senX3-R) and *regX3* transcript (regX3-F/regX3-R), and the *senX3-regX3* co-transcript (SR-F/SR-R). The Tn insertion is at bp 162 in the *senX3* gene of *senX3*::Tn (grey triangle). (b) RT-PCR results. Lanes: 1100 bp DNA marker (Fermentas); 2, *senX3* expression in wild-type; 3, co-transcript expression in wild-type; 4, *regX3* expression in wild-type; 5, *senX3* expression in *senX3*::Tn; 6, co-transcript expression in *senX3*::Tn; 7, *regX3* expression in *senX3*::Tn.

(Fig. 5b). Conversely, a bright 192 bp band representing monocistronic *regX3* transcript was present in P_i-depleted *senX3*::Tn, further confirming that Mtb *regX3* induction is chiefly monocistronic during P_i depletion.

DISCUSSION

Although SenX3-RegX3 is one of the better characterized Mtb 2CRSs, much remains to be determined regarding its role in Mtb adaptation and virulence. In this study, we provided direct evidence that this 2CRS is responsive to both P_i depletion and nutrient starvation, which may be important conditions encountered by Mtb during latent tuberculosis infection in humans (Gomez & McKinney, 2004). In addition, we have shown that the MIRUs within the *senX3-regX3* IR have promoter activity, potentially driving *regX3* expression independently from *senX3* expression under the same conditions.

Accumulating evidence has suggested that SenX3-RegX3 is directly involved in the mycobacterial phosphate starvation response (Glover *et al.*, 2007; James *et al.*, 2012; Rifat *et al.*, 2009). P_i limitation is believed to be a physiologically relevant microenvironment encountered by Mtb within the arrested macrophage phagolysosome (Rengarajan *et al.*, 2005). Expression of *senX3* and *regX3* is rapidly induced in *M. smegmatis* under P_i-limiting conditions (Glover *et al.*, 2007), and a *senX3-regX3*-deficient mutant shows impaired growth during P_i depletion (James *et al.*, 2012). Similarly, the homologous Mtb 2CRS is upregulated in response to P_i depletion, and expression of the phosphate-specific transport operon *pstS3-pstC2-pstA1* is RegX3-dependent (Rifat *et al.*, 2009). In the current study, we found that the Mtb *senX3* promoter responds to P_i depletion.

Our data also demonstrate activity of the Mtb *senX3* promoter during nutrient starvation, in which P_i is in abundance. Our findings are consistent with the function of the homologous PhoBR 2CRS in *E. coli*, which regulates the transcriptional response to P_i depletion and nutrient starvation (Baek & Lee, 2007; Rao *et al.*, 1998). Prior studies have highlighted the importance of *senX3-regX3* in Mtb survival in murine lungs (Parish *et al.*, 2003; Rickman *et al.*, 2004; Tischler *et al.*, 2013). Previously, we have shown that this 2CRS is also required for long-term Mtb survival in the lungs of guinea pigs (Rifat *et al.*, 2009).

As in the case of other 2CRSs, RegX3 was shown to be autoregulatory in *Mycobacterium bovis* BCG and *M. smegmatis* (Glover *et al.*, 2007; Himpens *et al.*, 2000), binding to the promoter of *senX3*. The current study demonstrates that the IR between *senX3* and *regX3* may serve as a promoter directly driving monocistronic expression of Mtb *regX3* during axenic growth in nutrient-rich broth, as well as under P_i depletion and nutrient starvation. Interestingly, *M. smegmatis* RegX3 can be phosphorylated in the absence of SenX3 in phosphate-rich medium (Glover *et al.*, 2007). These data raise the question of whether RegX3 may be functionally independent of SenX3. We and others have shown previously that expression of the polyphosphate kinase gene *ppk1*, which is known to regulate synthesis of the stringent response alarmone, (p)ppGpp, through the *mprA-sigE-relA* signalling pathway (Sureka *et al.*, 2007), is RegX3-dependent (Rifat *et al.*, 2009; Sanyal *et al.*, 2013). Thus, RegX3 may serve as a master regulatory switch, providing a common means by which to transduce the signals of P_i depletion and nutrient starvation, thereby inducing the stringent response, which is critical for Mtb persistence in the host (Dahl *et al.*, 2003). The presence of multiple promoters to provide fine-tuning of gene expression is not

unusual within bacterial operons (Barry *et al.*, 1979; Fornwald *et al.*, 1987; Taylor *et al.*, 1984). For example, in addition to the principal promoter (Dorman, 1995), which regulates the transcription of the DNA gyrase genes, the Mtb operon containing the essential genes *gyrB* and *gyrA* also includes a promoter within the intergenic region between these two genes (Unniraman *et al.*, 2002).

Interestingly, we observed strong promoter activity for pIR-2 under nutrient starvation, but our data suggest that expression of Mtb *regX3* appears to be mainly bicistronic under this condition. One possible explanation for these discrepant findings is that the promoter activity of the *senX3-regX3* IR may be activated (or de-repressed) during P_i depletion but repressed during nutrient starvation. The pIR-2 clone contained flanking sequences of 16 bp from the 3′ end of *senX3* and 40 bp from the 5′ end of *regX3*, which may not constitute the entire native regulatory sequences of this promoter region. Putative activators and repressors of the promoter activity of the *senX3-regX3* IR remain to be identified. In addition, much remains to be determined regarding regulation of Mtb *senX3-regX3* co-expression. This operon has been shown to be a member of the sigma factor SigC regulon, since these genes are downregulated in a *sigC*-deficient mutant (Sun *et al.*, 2004), although their regulation may be indirect. Recent data indicate that expression of this 2CRS is negatively regulated by the Pst system component PstA1, since a *pstA1* deletion mutant exhibited increased expression of *regX3*, which was responsible for sensitization of Mtb to nitric oxide synthase (NOS2)-dependent killing mechanisms in the lungs of mice (Tischler *et al.*, 2013).

The *senX3-regX3* IR is found in all members of the Mtb complex and *Mycobacterium leprae*, but not in other mycobacterial species. It is composed of a variable number of MIRUs, with the vast majority of Mtb strains containing two full 77 bp MIRUs followed by one partial unit of 53 bp (Magdalena *et al.*, 1998b). In our study we were unable to evaluate the promoter activity of the intact Mtb *senX3-regX3* IR. Sequence analysis of this group of mycobacteria found that a short duplication of DTGA is located in *M. leprae* at the site where an MIRU is present in Mtb, indicating the possibility of excision and insertion of MIRUs in the Mtb genome (Supply *et al.*, 1997). Consistent with such a scenario, each of the clones we studied contained varying combinations of MIRUs with DTGA sites at each end. However, the precise mechanism underlying MIRU excision and insertion requires further study.

MIRUs differ from other small repetitive DNA sequences in that they lack obvious palindromic sequences permitting stable secondary structures, they are direct tandem repeats, their orientation is in the same direction relative to transcription of adjacent genes, and they often contain small ORFs, whose initiation and stop codons overlap stop and initiation codons of adjacent repeat units (Supply *et al.*, 1997). A BLAST search using the sequence of the Mtb CDC1551 *senX3-regX3* IR revealed 40–50 regions throughout the Mtb genome containing MIRUs. The majority of these contain a partial unit or single unit with approximately 90 % homology to the *senX3-regX3* MIRU. Several additional regions contain two to three units with ~70 % homology and three regions contain a maximum of four full units and a partial unit with ~60 % homology to the MIRU within the *senX3-regX3* IR. Interestingly, genes flanking those MIRUs mostly encode enzymes, such as ATP-dependent DNA helicase, ABC-type efflux protein, zinc-binding dehydrogenase and enoyl-CoA hydratase, and few encode transcriptional regulators, such as the FtsK/SpoIIE family protein and methyltransferase-related protein. Overall, those MIRUs appear to disseminate by transposition into DTGA sites and have at least 60 % homology with variable copy numbers, suggesting a common origin but evolutionary modification to suit the function of flanking genes (Supply *et al.*, 1997). A BLAST search revealed that an identical match to the *senX3-regX3* IR sequence is lacking in the rest of the Mtb genome. We specifically searched the IR of the other known Mtb 2CRSs, as well as the upstream regions of putative orphan HKs and RRs, and identified a 36 bp fragment with 89 % homology to the *senX3-regX3* MIRU within the IR of the *mtrB-mtrA* operon, which is essential for Mtb growth (Via *et al.*, 1996; Zahrt & Deretic, 2000). In this study, we found that the number of MIRUs was critical to promoter activity of the IR in response to P_i depletion and nutrient starvation. Therefore, the potential regulatory function of homologous Mtb MIRUs under phosphate depletion or nutrient starvation, as well as other experimental conditions, merits further study.

Whether RegX3 regulates its own expression through binding at the IR, and whether SigC and PstA1 can regulate *regX3* expression independently of *senX3* remains to be determined. Since RegX3 has been implicated in Mtb virulence, it is possible that the observed variability in virulence between mycobacterial species and Mtb clinical isolates is at least partially attributable to differences in the number of intact MIRUs in the *senX3-regX3* IR. Interestingly, *M. bovis* BCG lacks a 53 bp MIRU in the *senX3-regX3* IR, which is present in other *M. bovis* strains, as well as Mtb, *Mycobacterium microti* and *Mycobacterium africanum* (Magdalena *et al.*, 1998b). Furthermore, BCG containing two 77 bp MIRUs in the *senX3-regX3* IR showed greater immunogenicity and persistence in a mouse model than BCG containing one MIRU (Magdalena *et al.*, 1998b).

Our findings challenge our previous understanding of the *senX3-regX3* 2CRS as simply an auto-regulatory, bicistronic operon, although further studies are required to elucidate the regulatory mechanisms governing bicistronic and monocistronic transcription of each of these genes. Our study also highlights the potential regulatory role of MIRUs in driving expression of downstream genes. Based on our findings, the role of single or half-unit MIRUs distributed throughout the Mtb genome deserves further study. Future studies in our laboratory will focus on elucidating the precise *regX3* promoter region within the IR, as well as identifying positive and negative regulators of *regX3* expression. In addition,

the independent contribution of *senX3* and *regX3* to Mtb virulence and persistence in the host is being actively investigated.

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