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Deletion of SenX3–RegX3, a key two-component regulatory system of *Mycobacterium smegmatis*, results in growth defects under phosphate-limiting conditions

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Two component regulatory systems are key elements in the control of bacterial gene expression in response to environmental perturbations. The SenX3–RegX3 system is implicated in the control of phosphate uptake in *Mycobacterium smegmatis* and *Mycobacterium tuberculosis. regX3* is reported to be essential in *M. smegmatis*, but not in *M. tuberculosis*. We attempted to construct complete *senX3–regX3* operon deletion strains of *M. smegmatis*; initially we found that the operon could only be deleted when another functional copy was provided. Using a strain in which the only functional copy of the operon was present on an integrating plasmid, we attempted to replace the functional copy with an empty vector. Surprisingly, we obtained strains in which the functional copy had been deleted from the chromosome at a low frequency. We deleted the *senX3* gene in a similar fashion, but it was not possible to delete *regX3* alone. To identify possible compensatory mutations we sequenced the whole genome of two deletion strains and the wild-type. A synonymous single nucleotide polymorphism (SNP) in a lipoprotein was found in all deletion strains, but not the parental strains, and a frameshift mutation in *nhaA* was identified in three of the four deletion strains. Operon deletion strains were more sensitive to phosphate limitation, showing a reduced ability to grow at lower phosphate concentrations. The *M. tuberculosis* operon was able to functionally complement the growth phenotype in *M. smegmatis* under phosphate-replete conditions, but not under low phosphate conditions, reinforcing the difference between the two species. Our data show that, in contrast with previous reports, it is possible to delete the operon in *M. smegmatis*, possibly due to the accumulation of compensatory mutations, and that the deletion does affect growth in phosphate.

INTRODUCTION

Two-component regulatory systems (TCRs) are a common bacterial mechanism for effecting changes in global regulation in response to specific environmental stimuli (Stock & Guhaniyogi, 2006). Mycobacteria have a number of classical TCRs (Cole *et al.*, 1998); for most systems the environmental signal is detected by a sensory protein which first autophosphorylates a conserved histidine, and then transfers the phosphate to the response regulator. Normally phosphorylation of the regulator leads to activation of its DNA-binding activity resulting in

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Abbreviations: DCO, double cross-over; SCO, single cross-over; SNP, single nucleotide polymorphism; TCR, two-component regulatory system.

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induction or repression of the target genes (Stock & Guhaniyogi, 2006).

The SenX3–RegX3 system of mycobacteria has been studied in some detail. In *M. tuberculosis*, the system is required for virulence, with mutant strains showing attenuation in macrophage and murine infection models (Parish *et al.*, 2003; Rickman *et al.*, 2004; Rifat *et al.*, 2009). In contrast, the regulator, RegX3, is essential in the non-pathogenic species *Mycobacterium smegmatis*, although deletions of the sensor (SenX3) are viable (Glover *et al.*, 2007). The reason for this difference is not known, although at least one of the genes controlled by RegX3 in *M. smegmatis* (*phoA*) is absent from *M. tuberculosis* (Cole *et al.*, 1998; Glover *et al.*, 2007). There is some genetic variation in the SenX3-RegX3 locus across mycobacterial species. All *Mycobacterium bovis* BCG strains sequenced have non-synonomous mutations in both SenX3 and RegX3, which is likely to affect the function of both proteins (Garcia Pelayo *et al.*, 2009). In addition, variation in the number of repeating units located between the two genes are seen in clinical isolates of *M. tuberculosis* (Supply *et al.*, 1997).

The SenX3 TCR is involved in the response to phosphate limitation, although it is unlikely that the direct stimulus for SenX3 is phosphate concentration (Gebhard & Cook, 2008; Glover *et al.*, 2007; Rifat *et al.*, 2009). Depletion of phosphate leads to upregulation of the *senX3–regX3* operon, as well as two systems involved in phosphate uptake, the high-affinity PstSCAB and PhnDCE phosphate transport systems (Gebhard & Cook, 2008; Glover *et al.*, 2007; Rifat *et al.*, 2009). In *M. smegmatis* expression of the PstSCAB systems is controlled directly by SenX3–RegX3 (Glover *et al.*, 2007), whereas PhnDCE expression is directly controlled by the regulator PhnF (Gebhard & Cook, 2008) and indirectly controlled by the SenX3 system through its action on PhnF (Gebhard & Cook, 2008).

In *M. tuberculosis* the SenX3 system is part of a larger regulatory complex involving SigC and MtrA. SenX3 expression is dependent on SigC, such that it is significantly downregulated in a SigC deletion strain (Sun *et al.*, 2004). A number of direct interactions with promoter regions are known for the *M. tuberculosis* regulator RegX3. Binding of purified RegX3 to the promoter regions of *ald*, *gltA1* and *cydB* as well as its own promoter has been demonstrated (Himpens *et al.*, 2000; Roberts *et al.*, 2011). Interactions have also been detected with the promoter regions of Rv0353, Rv3132-3134 (*dosSR*), *rubAB*, *groEl1*, *ufaA2* and *whiB7* using a bacterial one-hybrid system (Guo *et al.*, 2009).

METHODS

Culture of mycobacteria. *M. smegmatis* mc^{2}155 was grown at 37 °C in Lemco medium (10 g peptone, 5 g Lemco powder, 5 g NaCl, all l^{-1}) containing 0.05 % (w/v) Tween 80 for liquid cultures or 15 g agar l^{-1} for solid medium. Hygromycin was used at 100 μg ml^{-1}, gentamicin at 20 μg ml^{-1}, kanamycin at 20 μg ml^{-1} and sucrose at 10 % (w/v) where required. The low phosphate medium contained 20 mM MOPS pH 6.6, 0.5 g $MgSO_4$, 2 mg $ZnCl_2$, 10 mg $FeCl_3$, 0.5 mg $CuCl_2$, 0.5 mg $MnCl_2$, 0.5 mg $Na_2B_4O_7$, 0.5 mg $(NH_4)_4Mo_7O_{24}$, all l^{-1}, and 30 mM $(NH_4)_2SO_4$ supplemented with 2 % glucose, 5 % BSA and 0.05 % Tween 80; phosphate was added to the required concentrations as K_2HPO_4 : KH_2PO_4 pH 6.6; cultures were grown at 37 °C with shaking at 100 r.p.m.

Construction of plasmids. A deletion delivery vector for the entire SenX3–RegX3 region was constructed. The upstream and downstream regions were amplified by PCR using primer pairs Del1/Del2 (5′-CCCCGGTACCCAGTAACGCCGACACCAAG and 5′-CCCAAGCTTAGCCAGTGGAACGAGAAGAA) and Del3/Del4 (5′-CCCGGATCCCCACCACGAGAAGATGGACT and 5′-CCCCGGTACCGCCTGGGATACAAGCTGGAG); the fragments were cloned into p2NIL (Parish & Stoker, 2000) using the underlined restriction sites as *Hin*dIII–*Kpn*I and *Kpn*I–*Bam*HI fragments. The *Pac*I cassette from pGOAL19 (Parish & Stoker, 2000) carrying *hyg*, *lacZ* and *sacB* was cloned in to this vector to generate the deletion delivery plasmid pELMO2.2.

The complementing plasmid pSOUP211.1 was generated by cloning a 2.3 kb fragment carrying the complete *M. tuberculosis senX3–regX3* operon together with 179 bp of the upstream region carrying the promoter into pINT3, a single copy integrating vector carrying gentamicin resistance (Parish *et al.*, 2005). Inverse PCR was used to generate plasmids with deletions of either *senX3* or *regX3*: primer pair isen1/isen2 (5′-GCATATGCGAGGCCTATCACGACGAGC and 5′-GCATATGAACAGCGCCGAGAACACAGT) was used to generate a 1.1 kb deletion in *senX3*; primer pair ireg1/ireg2 (5′-GCATATGCTCGTCCTCCACAATCAACA and 5′-GCATATGTAACCCGGTTCACTTGGTGA) was used to generate a 0.6 kb deletion in *regX3*; an *Nhe*I site was incorporated into each primer to facilitate rejoining of the plasmid ends.

Construction of recombinant strains. The deletion delivery vector pELMO2.2 was pre-treated with UV (Hinds *et al.*, 1999) and electroporated into *M. smegmatis* to generate single cross-overs (SCOs). The genotype of one SCO was confirmed by Southern blot analysis and this strain was streaked onto antibiotic-free medium to allow the second cross-over to occur. Double cross-overs (DCOs) were selected as white colonies appearing on sucrose-containing medium. Colony PCR was used to determine which allele (wild-type or deletion) was present using primers Check1/Check2 (GACCTGCACTGGTACTGCAC and GACCTGCACTGGTACTGCAC) which amplify 2.3 kbp for the wild-type allele and 0.4 kbp for the deletion allele.

A merodiploid carrying the *M. tuberculosis* operon was constructed as follows: a plasmid carrying the complete *M. tuberculosis* SenX3–RegX3 operon (pSOUP211.1) was transformed into the SCO strains. DCOS were generated by streaking onto gentamicin-only medium, followed by selection on sucrose-containing medium. DCOs were screened by PCR as before.

Switching was carried out as described previously (Pashley & Parish, 2003). Briefly, strains were electroporated and selection for the incoming vector used to isolate transformants. Transformants were patched onto the relevant antibiotic plates. PCR primers gen1/gen2 (5′-GGCTCAAGTATGGGCATCAT and 5′-TCGTCACCGTAATCTGCTTG) and kan1/kan2 (5′-CGAGCATCAAATGAAACTGC and 5′-GAGCCATATTCAACGGGAAA) were used to screen for the presence of the gentamicin and kanamycin resistance cassettes respectively.

Analysis of *regX3* expression. RNA was isolated from *M. smegmatis* and cDNA was synthesized using random primers. Quantitative PCR was carried out using primer/probe combinations for real-time (Taqman) PCR. For *M. smegmatis regX3*, primers 5′-CCGACGTGTGCAAGCAGTT and 5′-TGACATAGTCGTCAGCACCGA were used with the probe 5′-TGACGGCGCGCGACAGCGA.

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For *M. tuberculosis regX3*, primers 5′-ATCTGATGCTGCCTGGGATGT and 5′-ACCACCTTGTCGATCTCGCTAT were used with the probe 5′-TGCGCGCTCGGTCCAGCGT. For *M. smegmatis sigA*, primers 5′-AAGCGGGCAGCCAAGAG and 5′-TCGAGATCGTCGGTCACCTCAA were used with probe 5′-TTGGCGGCCCGCTTGGCC.

Whole genome sequencing. Genomic DNA was prepared from *M. smegmatis* strains and whole genome sequencing carried out as previously described (Ioerger *et al.*, 2010). SNPs were confirmed by PCR amplification and sequencing.

RESULTS AND DISCUSSION

Construction of a *senX3–regX3 operon* deletion in *M. smegmatis*

We are interested in the role of the SenX3–RegX3 two component regulatory system in mycobacteria. We had previously constructed a deletion strain of *M. tuberculosis* in which we removed the only copy of *regX3* from the genome; this strain showed a growth defect *in vitro* (in axenic culture and in macrophages) and *in vivo* in the mouse model of infection (Parish *et al.*, 2003). Work with other deletion or transposon mutants of RegX3 and/or SenX3 has confirmed that this two component system is required for infection in mice and guinea pigs (Parish *et al.*, 2003; Rickman *et al.*, 2004; Rifat *et al.*, 2009).

In order to extend our studies we attempted to construct a deletion strain in *M. smegmatis*. During this work, other authors published the surprising observation that RegX3 was essential in *M. smegmatis* (Glover *et al.*, 2007), although it can be easily deleted from *M. tuberculosis* (Ewann *et al.*, 2002; Parish *et al.*, 2003; Rickman *et al.*, 2004; Rifat *et al.*, 2009). This suggests there are differences in the regulon between the two species which would benefit from a deletion strain. Glover *et al.* (2007) attempted to construct a *regX3* mutant and showed that it could only be achieved when complemented with a second copy. They also attempted to generate a *senX3–regX3* double deletion unsuccessfully, but in this case they did not show it could be achieved after complementation. In both cases, the mutants were marked with a kanamycin resistance gene, which could have polar effects. We attempted to construct an unmarked deletion of the complete operon to assess if it were truly essential. Using a two-step homologous recombination procedure (Parish & Stoker, 2000) we screened 183 DCOs; all were wild-type. When a merodiploid strain containing the functional *M. tuberculosis* operon was used, we were able to delete the entire chromosomal operon in 7/7 DCOs screened. Southern analysis confirmed that all had lost the wild-type copy of the operon (Fig. 1). This suggests that the operon is essential, and also indicates that the *M. tuberculosis* system is functional and able to complement for the *M. smegmatis* system. Unusually, all of the DCOs we screened were deletion alleles, suggesting that there may be a selective pressure against the presence of both systems simultaneously, possibly from interference.

These data support the work previously reported showing that the chromosomal operon is essential for growth

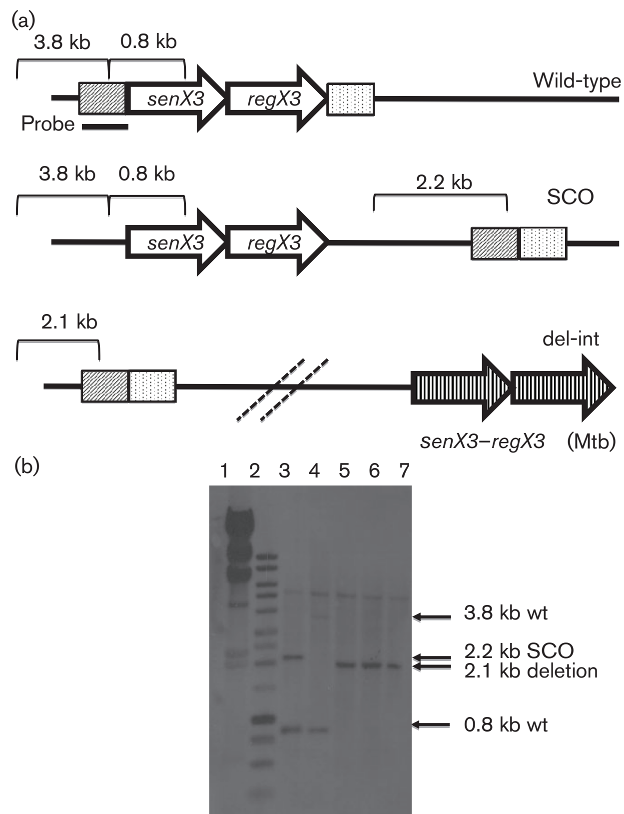

Fig. 1. Complementation of the *senX3–regX3* operon of *M. smegmatis* with the operon from *M. tuberculosis*. The operon deletion delivery vector was electroporated into wild-type *M. smegmatis* to generate the SCO strain. An integrating vector carrying the *M. tuberculosis senX3–regX3* operon was introduced into the SCO strain generating a merodiploid; DCOs in which the chromosomal copy of the operon was deleted were isolated in this background (del-int strains) but not in the wild-type background. (a) Expected genotypes for strains showing predicted sizes for hybridization with the *M. smegmatis senX3* upstream region probe for the wild-type, SCO and del-int strains. (b) Southern analysis of strains. Lanes: 1, 1 kb ladder; 2, Lambda *Hin*dIII ladder; 3, SCO strain; 4, wild-type strain; 5–7, del-int strain carrying the *M. tuberculosis* operon only. The wild-type, deletion and SCO bands are indicated. Cross-hybridization of the upstream probe to another region was noted in all strains (weak band at 4.5 kb); this was not seen with other probes (data not shown). Note that the *M. smegmatis* probe does not hybridize to the *M. tuberculosis* operon.

(Glover *et al.*, 2007). However, in the previous experiments, no attempt to remove the second functional copy was made after the chromosomal deletion was constructed. In order to address this omission, we attempted to remove the integrated copy of the operon; we made use of our switching strategy in which the resident integrated vector (carrying the operon) is replaced by a newly transformed control vector (no operon) simply by selecting for the

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resistance marker on the incoming plasmid (Pashley & Parish, 2003) (Fig. 2).

The del-int strain of *M. smegmatis* (deletion of chromosomal operon, integrated copy of the *M. tuberculosis* operon) with the genotype *senX3*$_{Msm}$Δ *regX3*$_{Msm}$Δ [*senX3*$_{Mtb}$ *regX3*$_{Mtb}$ Gm L5 *int*] was electroporated with either pSM128 (streptomycin resistance) (Dussurget *et al.*, 1999) or pKP186 (kanamycin resistance) (Springer *et al.*, 2001) and the incoming vector was selected for. We obtained a very low frequency of transformants which had lost gentamicin resistance but gained either streptomycin or kanamycin resistance, indicating that plasmid switching had occurred. PCR and Southern analyses confirmed the loss of the *M. tuberculosis* operon, and the chromosomal deletion of the *M. smegmatis* operon (Fig. 2).

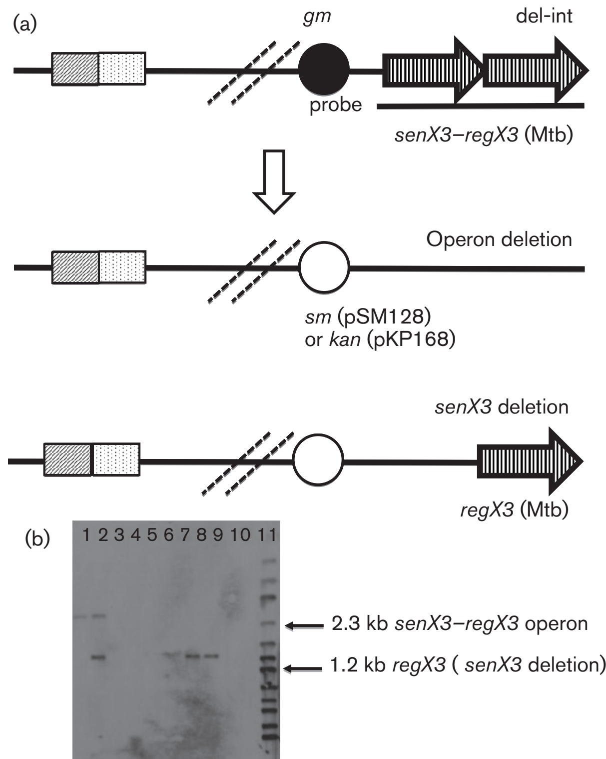

Fig. 2. Deletion of the *senX3–regX3* operon in *M. smegmatis* by gene switching. (a) Schematic of the gene switching with expected genotypes for strains. The del-int strain was electroporated with pSM128 (*sm*) or pKP186 (*kan*) integrating vectors and selected on the appropriate antibiotic. The del-int strain was also transformed with a vector carrying either SenX3(Mtb) or RegX3(Mtb) alone. Streptomycin- or kanamycin-resistant strains were isolated and replacement of the resident vector (carrying the *senX3–regX3* operon from *M. tuberculosis*) was confirmed by loss of gentamicin resistance. Switched strains were obtained for the operon deletion (pSM128/pKP186; SenX3Δ, RegX3Δ) and the RegX3-only plasmid (pSNUFFY1: SenX3Δ, RegX3$_{Mtb}$), but not the SenX3-only plasmid (pSNUFFY2: SenX3$_{Mtb}$, RegX3Δ). (b) Southern analysis of strains hybridized to the *M. tuberculosis* operon probe (indicated). Lanes: 1, 1 kb ladder; 2, empty; 3, wild-type *M. smegmatis*; 4, merodiploid; 5, del-int strain; 6–7, empty plasmid pKP186 (operon deletion); 8–9, empty plasmid pSM128 (operon deletion); 10, cointegrant containing complete operon and pSNUFFY1 plasmid; 11, pSNUFFY1 RegX3 plasmid (*senX3* deletion). Note that the *M. tuberculosis* probe does not hybridize to the *M. smegmatis* operon.

We also attempted to delete *senX3* and *regX3* individually using the same strategy. Plasmids carrying either *senX3* alone or *regX3* alone were switched in to the del-int strain. A control plasmid with the complete operon successfully switched. A low number of transformants were obtained for both plasmids; on patching and PCR analysis, we determined that the RegX3 only vector had replaced the operon in some of the transformants (loss of gentamicin resistance, lack of wild-type PCR product, presence of deletion PCR product). Southern analysis of a switched (deletion) strain and a cointegrant, in which both plasmids were resident, is shown (Fig. 2). In contrast the SenX3 only vector could not replace the operon and all transformants had the wild-type operon. Thus we were able to delete *senX3* independently, but not *regX3* as predicted. In the *senX3* deletion strain, *regX3* should still be expressed from the same (native) promoter as for the operon. We confirmed this by RT-qPCR; *M. tuberculosis regX3* mRNA was seen in this strain (relative expression to *sigA* of 0.2), but not in the wild-type strain (zero expression). *M. smegmatis regX3* was seen in the wild-type strain (relative expression to *sigA* of 0.2), but not in the deletion strain (zero expression).

The isolation of an operon deletion was surprising, given previous data suggesting that it was not possible, as well as our own data confirming the essentiality of *regX3*. Using the same method we were able to construct *senX3* deletions, but not *regX3* deletions. It is not immediately obvious why were able to obtain a deletion mutant using the switching method, but not with a two-step homologous recombination method. We characterized one of the mutant strains carrying the empty vector pKP186 (genotype *senX3*$_{Msm}$Δ *regX3*$_{Msm}$Δ [kan, L5 *int*]) carefully to confirm it did indeed lack the operon; Southern analysis using the *M. smegmatis* and the *M. tuberculosis* operons as probes confirmed their absence (Fig. 2). PCR amplification of the resistance markers in the integrating vectors confirmed that the gentamicin marker had been lost (data not shown). Cultures of the mutant strains (10^8 c.f.u.) were plated onto gentamicin to confirm that all cells were sensitive and that there was no residual population carrying the operon plasmid. One further possibility to account for our inability to obtain a mutant using the two-step method could be that the mutants are sucrose-sensitive, since the mutant isolation method involved plating onto sucrose. We tested the ability of the deletion strain to grow on plates containing sucrose, but it was fully viable and showed no defects.

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Table 1. SNPs in strains of *M. smegmatis*

SNPs identified in recombinant *M.smegmatis* strains. Whole genome sequencing was used to identify SNPs in two operon deletion strains (1 : 1 and 2 : 1). Mutations that were found in both deletion strains are shown. SNPs were confirmed by amplification and sequencing of the locus in all strains including the wild-type, SCO, SenX3 deletion strain (Mutant 1) and SenX3–RegX3 deletion strains (Mutants 1 : 1, 2 : 1, 1 : 2 and 2 : 2). nt, Not tested; –, no frameshift.

Gene ID	Gene	Genome location	SNP	Wild-type	SCO	SenX3Δ Mutant 1	SenX3–RegX3Δ			
							Mutant 1 : 1	Mutant 2 : 1	Mutant 1 : 2	Mutant 2 : 2
Both strains										
MSMEG_0935	Upstream	1016382	Non-coding	T	A	A	A	A	A	A
MSMEG_0938c	CHP	1018768	sSNP Ser	G	A	A	A	A	A	A
MSMEG_3436	Toxin	3506652	Gly12 Val	G	T	T	T	T	T	T
MSMEG_3851	*lppI*	3922464	sSNP Gly	G	G	A	A	A	A	A
Mutant 1										
MSMEG_2775	*nhaA*	2841396	Frameshift	–	–	T	T	–	T	T
MSMEG_4064	Alcohol dehydrogenase	4137385	sSNP His118	G	nt	nt	A	G	nt	nt
MSMEG_4133	Transposase	4214324	Downstream	G	nt	nt	T	G	nt	nt
MSMEG_4133	Transposase	4214329	Downstream	C	nt	nt	A	C	nt	nt
MSMEG_4133	Transposase	4214350	Downstream	A	nt	nt	C	A	nt	nt
Mutant 2										
MSMEG_0144c	HNH endonuclease	167650	sSNP Glu	T	nt	nt	T	C	nt	nt
MSMEG_0144c	HNH endonuclease	167668	His270 Gln	G	nt	nt	G	C	nt	nt
MSMEG_3504	MP	3567074	Asp75 Glu	C	nt	nt	C	A	nt	nt

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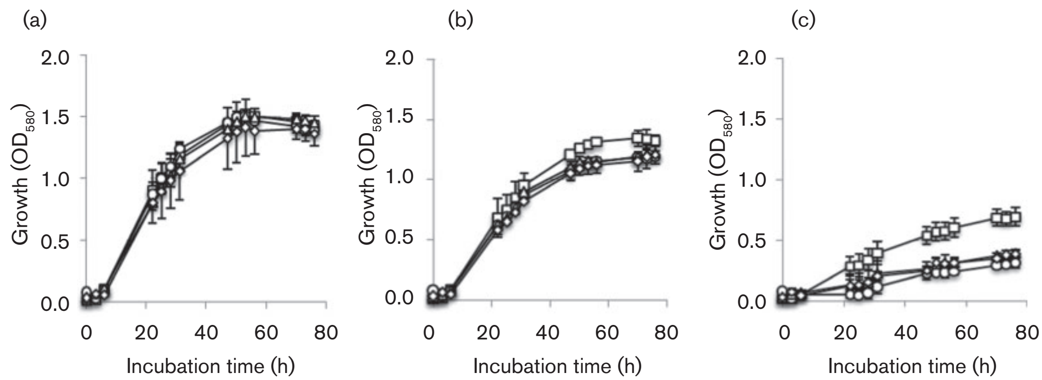

Fig. 3. Phosphate-limited growth of *senX3–regX3* deletion strains of *M. smegmatis.* Strains were grown in varying concentrations of phosphate: (a) 1 mM, (b) 100 μM, (c) 10 μM phosphate. □, Wild-type; ○, Shera 1 : 1 (operon deletion); △, Shera 2 : 1 (operon deletion); ◇, Kat1 (*senX3* deletion). Results are the mean ± SD of three independent cultures.

SenX3–RegX3Δ strains have a synonymous single nucleotide polymorphism (SNP) in *lppI* and a frameshift in *nhaA*

The *senX3–regX3*Δ strains were isolated at a low frequency leading us to hypothesize that a compensatory mutation had occurred allowing the deletion of the regulatory system. If RegX3 were required for the induction of expression of an essential gene under *in vitro* conditions, then a mutation which resulted in constitutive or dysregulated expression would lead to RegX3 being dispensable. Alternatively if RegX3 repressed the expression of a toxic gene, then mutation to disrupt the gene function would have a similar effect.

In order to determine if the deletion strains had compensatory mutations, we sequenced the complete genome of two isolates and the wild-type. A comparison of the three strains revealed a number of SNPs (Table 1), which were confirmed by amplification and sequencing of each locus. Four mutations that differed from wild-type were found, but three of these were also present in the SCO (MSMEG0935, MSMEG0938, MSMEG3436). Since we were unable to construct a mutant from the SCO, we discounted these as potential compensatory mutations. Only one mutation (MSMEG 3851, *lppI*) was seen in both strains; we sequenced this locus in two additional operon deletion strains and the SenX3 deletion strain and found that all strains had the same SNP which was not seen in the SCO or wild-type. However, this is a synonymous SNP in G138 (GGG to GGA). Changes in codon usage can affect translational efficiencies and subsequent levels of proteins, but in this case the codon usage for GGG or GGA is similar, suggesting that there would be little effect on protein levels.

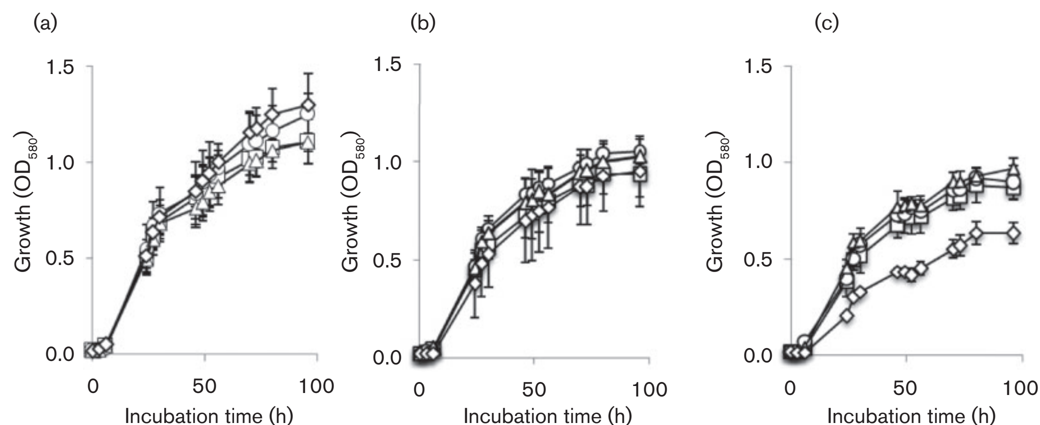

Fig. 4. Phosphate-limited growth of *senX3–regX3* deletion strains of *M. smegmatis.* Strains were grown in varying concentrations of phosphate: (a) 1 mM, (b) 100 μM, (c) 10 μM phosphate. □, Wild-type; ○, SCO; △, merodiploid; ◇, del-int strain (carrying copy of *M. tuberculosis* operon only). Results are the mean ± SD of three independent cultures.

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Of the remaining mutations, none was held in common by the two fully sequenced strains; strain 1 : 1 had four SNPs and strain 2 : 1 had three SNPs. We sequenced several of these mutations in the two additional operon deletion strains and the SenX3 deletion strains. We found that the mutation in *nhaA* was seen in all of these strains bar one. Since we had observed the same mutation in *nhaA* in four of the deletion strains, but it was not present in the SCO, we assumed that this SNP had arisen during culture of the merodiploid for transformation. Although not all of the deletion strains had the mutation, we considered this to be a good candidate as a compensatory mutation; presumably in the strain without an *nhaA* mutation, another compensatory mutation existed, possibly in either an endonuclease (MSMEG_0144c) or a membrane protein (MSMEG_3504).

The SNP in *nhaA* leads to a frameshift and so would inactivate its function. This suggests that lack of RegX3 may lead to uncontrolled expression of *nhaA* with lethal consequences for the cell. Thus we propose that RegX3 acts as a negative regulator of NhaA expression. Sequence similarity suggests that NhaA is a sodium–proton antiporter which is responsible for the exchange of Na^{+} and H^{+} across the cytoplasmic membrane, resulting in net export of sodium from the cell (Padan *et al.*, 2004). In *E. coli* NhaA plays a key role in pH homeostasis, maintaining the intracellular pH between determined levels regardless of the external environment. Dysregulation of the system could lead to increased import of protons and a drop in intracellular pH.

M. tuberculosis does not have a homologue of *nhaA* which could account for the difference in the essentiality of RegX between the two species, since RegX3 is not required to repress its transcription. *M. tuberculosis* has predicted homologues of KefB, a potassium antiporter, and ChaA, a calcium antiporter, suggesting subtle differences in pH homeostasis. Interestingly KefB is upregulated in *M. tuberculosis* during phosphate limitation, suggesting that it may be another member of the RegX3 regulon in *M. tuberculosis* (Rifat *et al.*, 2009). KefB plays an important role during macrophage infection, since mutants of *M. bovis* BCG fail to prevent phagosome acidification (Stewart *et al.*, 2005). *M. tuberculosis* is more susceptible to weak acids and is less able to maintain intracellular pH under acidic conditions (Zhang *et al.*, 2003), again reinforcing the differences between the two species.

SenX3 or RegX3 deletion strains show growth defects in low phosphate

The SenX3–RegX3 system is known to regulate the expression of several genes involved in phosphate accumulation in *M. smegmatis*, so we determined whether strains had growth defects under conditions of limiting P_i (Figs 3 and 4). As previously noted, the growth of *M. smegmatis* is limited when phosphate concentrations drop below 10 μM. We looked at growth under various concentrations of phosphate. We compared the growth of the two operon deletion strains and the *senX3* deletion strains to the wild-type. All strains grew similarly under phosphate-replete conditions (1 mM); however, the deletion strains showed a growth defect under phosphate-limiting conditions with little to no growth when phosphate was omitted from the medium. Growth of the deletion strains was retarded even at relatively high levels of phosphate (100 μM). These data confirm that RegX3-dependent changes in gene expression are required for the efficient uptake of phosphate under limiting conditions (Glover *et al.*, 2007; Rifat *et al.*, 2009).

We also assessed the ability of the merodiploid and del-int strain carrying the *M. tuberculosis* operon to grow under phosphate-limiting conditions. The merodiploid strain containing the operon from both species was able to grow as well as the wild-type under phosphate limitation. Surprisingly, the del-int strain was compromised for growth under low phosphate, demonstrating that the *M. tuberculosis* operon could not fully complement for its counterpart in *M. smegmatis*. These data also suggest that the essentiality of the operon in *M. smegmatis* is not related to its role in regulating phosphate uptake.

Conclusion

We demonstrated that deletion of the *senX3–regX3* operon is possible in *M. smegmatis* and suggest that this is due to compensatory mutations in the genome; there was no common nSNP in all four deletion strains we examined. However, we did identify an SNP in three strains and we propose that this mutation is compensatory and leads to the inactivation of the antiporter NhaA. It remains likely that there are other compensatory mutations that could allow the deletion of the operon. Our data reinforce the role of the SenX3–RegX3 TCR in responding to phosphate levels in the environment in *M. smegmatis*. The isolation of deletion strains in the operon, previously considered to be essential, provides a route forward to understanding the role of this global regulator in mycobacteria.

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