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# A two-component signal transduction system with a PAS domain-containing sensor is required for virulence of *Mycobacterium tuberculosis* in mice

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**Abstract**

*Mycobacterium tuberculosis*, the causative organism of tuberculosis, encounters oxidative stress during phagocytosis by the macrophage and following macrophage activation during an acquired immune response, and also from internally generated sources of radical oxygen intermediates through intermediary metabolism. We have identified the SenX3 protein, a sensor in 1 of the 11 complete pairs of two-component signal transduction systems in *M. tuberculosis*, as a possible orthologue of the Mak2p protein from the fission yeast *Schizosaccharomyces pombe* that is known to sense peroxide stress. Moreover, the SenX3–RegX3 two-component system was the top scoring hit in a homology search with the *Escherichia coli* ArcB–ArcA global control system of aerobic genes. Using structural modelling techniques we have determined that SenX3 contains a PAS-like domain found in a variety of prokaryotic and eukaryotic sensors of oxygen and redox. Mutants with knock-outs of *senX3* or of the accompanying transcriptional regulator *regX3* were constructed and found to have reduced virulence in a mouse model of tuberculosis infection, the mutant bacteria persisting for up to 4 months post-infection; complemented mutants had regained virulence confirming that it was mutations of this two-component system that were responsible for the avirulent phenotype. This work identifies the PAS domain as a possible drug target for tuberculosis and mutations in the *senX3–regX* signal transduction system as potentially useful components of live vaccine strains.


*Keywords:* Mycobacteria; Virulence; Sensor; PAS; Oxidative; Stress

The need for new approaches to assist in the control of tuberculosis (TB) is pressing. Annually 2–3 million people die of this disease and one-third of the world's population is believed to be infected with *Mycobacterium tuberculosis* [1]. Moreover, the synergy created between TB and AIDS makes each disease considerably more deadly [2]. The only vaccine for TB, *Mycobacterium bovis* BCG, the most widely used vaccine for any infectious disease and first used in the 1920s, does not provide adequate levels of protection in Africa, India, and some parts of the USA [3,4]. Added to this, the rise in drug-resistant strains of *M. tuberculosis* makes the identification of new drug targets and vaccines imperative. One approach to developing novel disease intervention strategies is to identify virulence determinants of *M. tuberculosis* which enable it to produce progressive infection. Such determinants could then be used as targets for the development of new therapeutic agents or for attenuated vaccine strains. Such strains should be unable to produce progressive infection but should

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persist in the host for a sufficient length of time to elicit a protective immune response.

Two-component signal transduction systems are used by many bacteria to allow them to respond rapidly to changes in their environment such as pH and temperature, and control a variety of bacterial processes including sporulation in *Bacillus subtilis* and chemotaxis in *Escherichia coli* [5]. In the simplest form, the two-component signal transduction system consists of a membrane bound sensor histidine kinase, which in response to a defined environmental stimulus undergoes autophosphorylation on a conserved histidine residue, using ATP as the phosphodonor. The phosphoryl group is then transferred to a conserved aspartate residue on the downstream response regulator which is thereby activated as either a positive or negative transcriptional regulator. Two-component signal transduction systems are mechanisms of regulating gene expression which appear nearly ubiquitous in bacteria, but have also been identified in some lower eukaryotes and plants.

From the point of view of designing attenuated vaccine strains the advantage of making mutants of two-component systems is that it is likely that these genes are only expressed at certain times in the growth of the pathogen. Ideally, vaccine strains should be unable to produce progressive infection but should persist in the host for a sufficient length of time to elicit a protective immune response. Since some two-component systems may regulate bacterial gene expression only during certain stages of the interaction with the host, mutants deficient in such systems might still survive within the host long enough to elicit an immune response before becoming growth-limited due to the mutation. This appears to be the case with the *M. bovis* vaccine strain BCG; it is not immediately eliminated from the host after vaccination.

The bacterial two-component family of histidine kinases and response regulators also provides a potential new target for novel drug therapies against microbial infection, largely due to the following features of such systems: (i) significant homology is shared among histidine kinase and response regulator proteins of different genera of bacteria, particularly in those amino acid residues located near active sites, therefore a single drug therapy may prove effective against a range of bacterial species; (ii) many pathogenic bacteria use two-component signal transduction systems to regulate expression of essential virulence factors that are required for survival within the host; (iii) bacteria contain many two-component systems, some of which are essential for viability, and (iv) signal transduction in mammals occurs by a different mechanism [6,7].

The genome of *M. tuberculosis* has been shown to encode 11 complete pairs of two-component signal transduction systems [8]; all but 4 have been deleted or are pseudogenes in the closely related intracellular pathogen *Mycobacterium leprae* [9]. The SenX3–RegX3 system is one of those four. Since the *M. leprae* genome has undergone extensive reductive evolution and is thought to represent a minimal complement of genes required for a mycobacterium to survive in an intracellular environment, the retention of SenX3–RegX3 supports its role as an important system for survival in the hostile host environment.

From homology searches we have identified the sensor protein SenX3 as a possible orthologue of a yeast two-component system sensing oxidative stress, and also this two-component system as a possible orthologue of the ArcB–ArcA system of *E. coli*, a global regulator of aerobic genes. Moreover, we have found that the SenX3 sensor contains a PAS domain that in other proteins is known to function as an input module that senses oxygen and redox potential. We have created null mutations in the sensor and regulator genes to investigate their role in the pathogenesis of *M. tuberculosis*. The resulting strains are indeed attenuated but show a persistence phenotype.

## Materials and methods

*Construction of senX3 and regX3 null mutants.* Approximately 1.9 kb of DNA sequence from each side of the *regX3* gene and 1.5 kb either side of the *senX3* genes were amplified with HotStarTaq (Qiagen) using the following primer pairs; for *regX3*, for the 5′ side Myc5 (5′-CTGGATCC-TATGGCGAAGAGCAGTT-3′) and Myc6 (5′-CTGGATCC-TCATCAGCGCCACTCTC-3′), and for the 3′ side Myc23 (5′-GCGGCCGC-TTGGCTCGGCGCAGCACATC-3′) and Myc24 (5′-GCGGCCGC-TTGTCTGCGGAGTGCTCATC-3′); for *senX3*, Myc279 (5′-GGATCC-GCTGATTCACGCTCATC-3′) and Myc280 (5′-GGATCC-AATCCGGTGAACGTCGC-3′) for the 5′ side and Myc297 (5′-GCGGCCGC-GTCAAACAGGTCACAAC-3′) and Myc282 (5′-GCGGCCGC-GTGCTGCAGAGCGCGGC-3′) for the 3′ side. The 5′ fragments were cloned into the *Bam*HI site and 3′ fragments into the *Not*I site of the suicide gene delivery vector p2NIL which is capable of replication within *E. coli* but lacks a mycobacterial origin of replication [10]. In the case of the *senX3* construct only, a hygromycin cassette was cloned into the *Kpn*I site of p2NIL to replace the *senX3* gene. A *Pac*I fragment from pGOAL19 containing the *lacZ* and *sacB* genes was inserted to give the completed suicide delivery vector pLR1 (*regX3*) or pDH1 (*senX3*). This vector contains the *sacB* gene from *Bacillus subtilis* that causes lethality when expressed in *M. tuberculosis* making it an effective counterselectable marker and a *lacZ* gene. The virulent *M. tuberculosis* strain H37Rv was transformed either with pLR1 and selection was made for potential single cross-overs as kanamycin-resistant colonies expressing the *lacZ* gene, or with pDH1 and selection made for kanamycin and hygromycin resistance. These colonies were streaked onto sucrose plates containing either no antibiotic selection (pLR1) or hygromycin resistance only (pMR25). Colonies showing the expected selection phenotype (kanamycin-sensitive, sucrose-resistant, and white due to the loss of the *lacZ* gene) were screened by PCR for potential double cross-overs. Genomic DNA isolated from potential double cross-over was used to confirm the genotype by genomic DNA microarray analysis, Southern blots, and DNA sequencing. For pLR1 (*regX3*), in 1 clone out of 12 tested the allelic exchange was shown to have resulted from a double cross-over event by Southern blot analysis, and confirmed by genomic microarray analysis and DNA sequencing; for pMR25, from the PCR

results all 12 appeared to be the result of a double cross-over event, one of which was confirmed by genomic microarray analysis and DNA sequencing.

*Complementation of the regX3 and senX3 mutations.* The complementing construct was prepared by PCR of genomic DNA using primers Myc420 (5′-ATCGAT-GCAGTCAGTTCAGCCAGGA-3′) and Myc423 (5′-TCTAGA-ACCAGACAGTCGCCAAGGTT-3′) and cloned into *Cla*I and *Xba*I sites of pKP186, an integrase-negative derivative of the integrating vector pMV306 [11] kindly provided by Dr. K.G. Papavinasasundaram. The resulting plasmid was sequenced completely to verify the absence of PCR-derived mutations. It was co-transformed into the *senX3* and *regX3* null mutants along with plasmid pBS-int carrying the integrase gene necessary to achieve integration of the plasmid into the chromosome. pBS-int lacks a mycobacterial origin of replication and is therefore lost from the bacterium. Attempts to achieve complementation with pMV306 constructs in which the integrase gene is carried on the same plasmid as the complementing genes were unsuccessful, possibly due to loss of the construct through action of the still present integrase gene.

*In vitro growth determination.* This was carried out in 1 litre polycarbonate culture bottles (Techmate) in a Bellco roll-in incubator (2 r.p.m.) at 37 °C in Dubos broth containing 0.05% Tween supplemented with 0.2% (v/v) glycerol and 0.04% (v/v) Dubos medium albumin. Optical density readings on aliquots removed were taken at 600 nm.

*In vivo growth characteristics of the ΔregX3 and ΔsenX3 mutants in a mouse model of TB infection.* The wild type and *regX3*- and *senX3*-deleted strains of *M. tuberculosis* H37Rv were grown as rolling cultures in Dubos broth (as above) to mid-exponential phase. Each strain was diluted in phosphate-buffered saline to give a suspension of approximately $10^6$ colony forming units (c.f.u.) per ml and 0.2 ml of these suspensions was inoculated intravenously into 6- to 8-week-old female Balb/c mice. The infection was monitored by removing the lungs and spleens of 3–5 infected mice at intervals. The tissues were homogenised by shaking with 2-mm-diameter glass beads in chilled saline with a Mini-Bead Beater (Biospec Products, Bartlesville, Oklahoma). Serial 10-fold dilutions of the resultant suspensions were plated onto Dubos 7H11 agar with Dubos oleic albumen complex supplement (Difco Laboratories, Surrey, UK). The numbers of colony forming units were determined after the plates had been incubated at 37 °C for approximately 20 days. The standard error of the mean was calculated. A paired *t* test was used to calculate that the means of the mutant samples were different at a given *p* value.

## Results

### *Search for a possible M. tuberculosis orthologue of a yeast two-component system sensing oxidative stress*

The identification of orthologous genes between the genetically well-characterised species of yeast and higher eukaryotes has been a profitable way of identifying genes in conserved aspects of the cell cycle [12]. We reasoned that the identification of prokaryotic orthologues to yeast genes could also be profitably used to identify genes necessary as virulence determinants. The fission yeast *Schizosaccharomyces pombe* has three histidine kinases, Mak1p, Mak2p, and Mak3p that are part of two-component systems that specifically sense peroxide stress [13]. Adjacent to the histidine kinase domain in these proteins are two (in the case of Mak1p) or one (in the case of Mak2p and Mak3p) copy of the input module which senses oxidative stress. The input module of Mak2p contains a PAS domain also identified in a variety of prokaryotic sensors of oxygen or redox, including other members of the histidine-kinase superfamily such as *E. coli* ArcB and Aer and *Rhizobium meliloti* FixL proteins [14]. The PAS domain in Mak2p is required for peroxide sensing (J. Quinn, B.A. Morgan, and J.B.A. Millar, unpublished data). We searched the *M. tuberculosis* proteome with the Mak2p protein using BLAST and found a possible orthologue in the SenX3 protein, the sensor histidine kinase (SwissProt Code SEX3_MYCTU) in the SenX3–RegX3 linked two-component signal transduction system [15].

### *Identification of the senX3–regX3 two-component system as a possible M. tuberculosis orthologue of the ArcB–ArcA aerobic global control system of E. coli*

The ArcB–ArcA global regulatory system controls the expression of genes coding for enzymes of aerobic metabolism, largely by repressing transcription of aerobic genes during anaerobic conditions but also by activation of a few genes [16]. BLAST searches with either the ArcA transcriptional regulator [17,18] or the ArcB sensor [19] identified the RegX3 and SenX3 proteins of *M. tuberculosis* as the top scoring hits, respectively (data not shown).

### *Identification of a PAS-like domain in SenX3 by structural modelling*

The histidine kinase domain in SenX3 spans residues 154–410 and includes a conserved histidine at position 167 that is the site of transphosphorylation to the receiver domain of the response regulator RegX3 [15]. Since we expected a sensor domain to be N-terminal to the transmitter domain but C-terminal to a transmembrane (TM) helix, we subjected the first 154 amino acids of SenX3 to transmembrane prediction using the program SOSUI [20]. This revealed a single TM helix between residues 3 and 25. We subsequently searched using sequences flanked by the transmitter domain and the TM helix (residues 29–153) which revealed weak similarities to the nitrogen fixation regulatory protein NifL from *Azotobacter vinelandii* [21] that contains two recognisable PAS domains, the second of which aligns to the query subsequence used in the database search (Fig. 1). Thus, it was possible that this region in the SenX3 sequence of *M. tuberculosis* could contain an atypical PAS domain. To test this, we carried out secondary structure prediction on the SenX3 and NifL sequences using the program PhD [22] which revealed a pattern of secondary structure reminiscent of the PAS domain from FixL (Fig. 1). FixL is an oxygen-responsive regulatory protein, for which the haem-binding PAS domain structure has been solved [23]. We modelled the


Fig. 1. Amino acid sequence alignments of the proposed PAS domains. The SenX3 proteins of *M. tuberculosis* (MYCTU), *M. leprae* (MYCLE), *M. bovis* (MYCBO), and *M. smegmatis* (MYCSM) are compared with those of the Nitrogen Fixation Regulatory Protein from *A. vinelandii* (NIFL_AZOVI) and the sensor protein FIXL from *R. meliloti* (FIXL_RHIME). α-Helical (dark shading) and β-strand (light shading) secondary structure prediction using PHD [22] is indicated for the top five sequences. The PAS domain of FIXL_RHIME has been solved [48] and the shading represents the actual secondary structure assigned by the program DSSP [49]. The secondary structure elements of the PAS domain are represented below the alignment (A–I). Note that the α-helical elements aD and aE are missing in the mycobacterial PAS domains.

three-dimensional structure of the N-terminal region of SenX3 to the PAS domain of FixL using the molecular modelling program QUANTA (Fig. 2). This demonstrated that the *M. tuberculosis* SenX3 protein has an atypical PAS domain in its N-terminal region in that it lacks two helices leading to a more open structure. Notably, a similar PAS domain could not be found in the other 10 *M. tuberculosis* two-component histidine kinases.

### *Deletion of the M. tuberculosis regX3 and senX3 genes*

In order to investigate the role of the *M. tuberculosis senX3–regX3* two-component signal transduction system, we have constructed *M. tuberculosis* strains with null mutations of the *senX3* gene and of the adjacent *regX3* gene coding for the transcriptional regulator, by deleting them from the chromosome as described in Materials and methods. The *senX3* construct removed

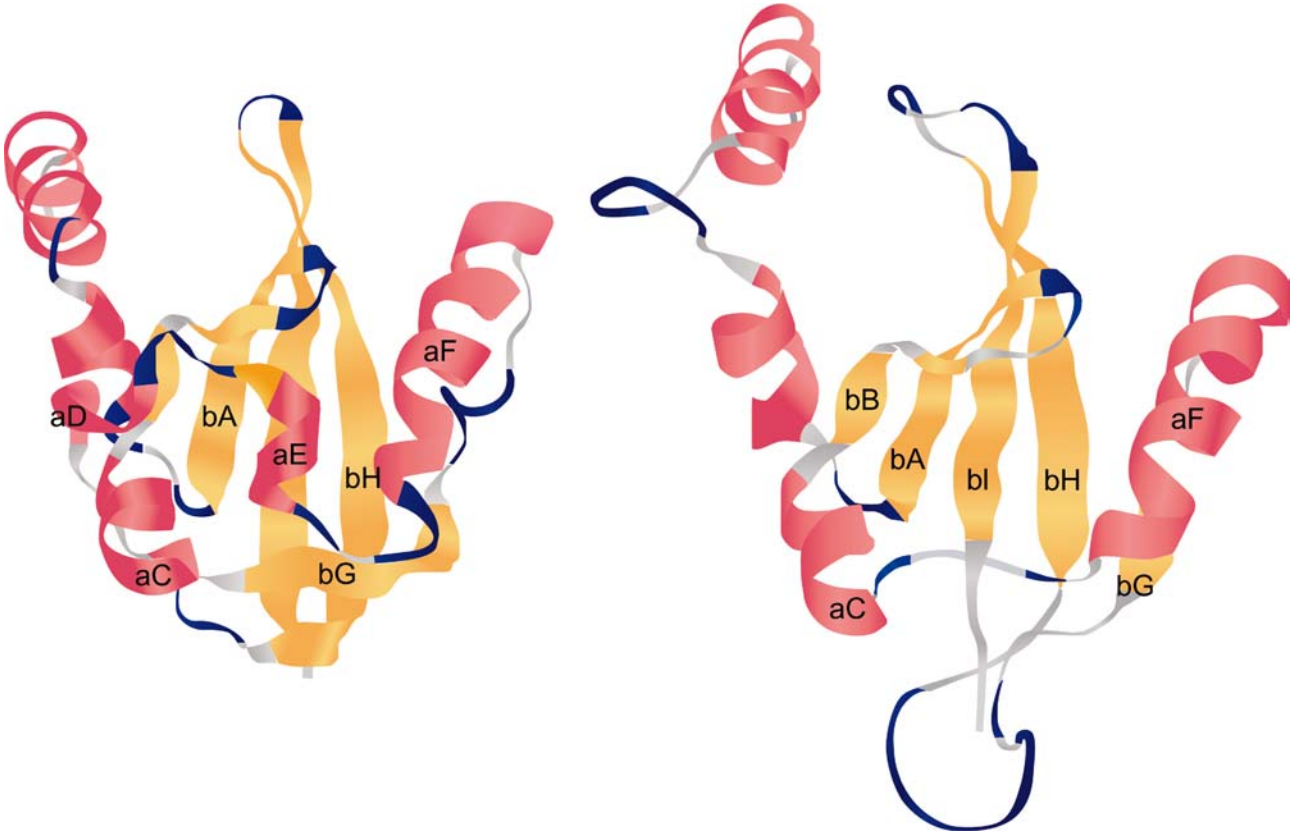


Fig. 2. Protein ribbon diagrams of PAS domains. The PAS domain from FIXL_RHIME (left) is compared with the modelled PAS domain from SENX3_MYCTU (right). Secondary structure is coloured red for helices and yellow for strands and labelled according to the sequence alignment in Fig. 1. Note the PAS domain from SENX3_MYCTU loses helices aD and aE leading to a more open structure.

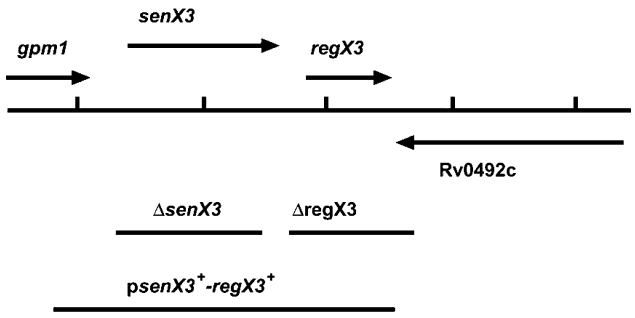


Fig. 3. Diagram of the genomic region of *M. tuberculosis* containing the *senX3–regX3* operon and adjacent genes, showing the extent of the deletions and the complementing plasmid p*senX3–regX3*. The marks on the chromosome are at 1000 bp intervals.

15 bp upstream of *senX3* and the whole of the coding region except for the carboxy-terminal 12 residues (Fig. 3). Since it had a hygromycin cassette in place of *senX3*, it was expected to have a polar effect on the *regX3* gene transcribed from the same mRNA. The *regX3* construct retained the complete upstream coding region of *senX3* but removed part of the intergenic region between *senX3* and *regX3*, the whole of the coding region of *regX3* and 78 carboxy-terminal residues of the adjacent gene Rv0492c transcribed on the opposite DNA strand. Therefore to ensure that the phenotypes of both mutants were due solely to the mutation of the *senX3* and *regX3* genes and not due to polar effects on adjacent genes or in the case of the *regX3* mutant, due to the removal of part of Rv0492c, complemented strains with wild type $senX3^+$ and $regX3^+$ alleles were made using the complete *senX3–regX3* region together with upstream sequences likely to carry the promoter, but without any of the adjacent gene Rv0492c, as described in Materials and methods. The complementing construct p$senX3^+$–$regX3^+$ contains the entire region between approximately half way through the upstream *gpm1* gene to 34 bp downstream of the end of the *regX3* coding region. It therefore contains all of the upstream region likely to contain the *senX3–regX3* promoter, all of the coding regions of these genes, but lacks the coding region of the adjacent gene Rv0492c (Fig. 3). Microarray experiments (data not shown) demonstrated that transcription of *regX3* was downregulated in the *ΔregX3* mutant but that transcription of both *senX3* and *regX3* was downregulated in the *ΔsenX3* mutant compared to the wild type, consequent upon the insertion of the hygromycin cassette in *senX3* having a polar effect on *regX3* expression and consistent with the known structure of the operon [15]. In the *ΔregX3* mutant two out of the three MIRUs (mycobacterial interspersed repetitive units) between the *senX3* and *regX3* genes were removed by the deletion; these could code for small peptides, although there is no experimental evidence for this [24].

*Growth of M. tuberculosis in vitro is largely unimpaired by deletion of the senX3 and regX3 genes*

Growth of the wild type H37Rv strain and both mutants was compared in vitro in rolling bottle cultures. The mutants grew at the same rate as the wild type H37Rv during exponential phase; the only difference from the wild type strain was that the *ΔregX3* mutant grew to a slightly lower optical density in stationary phase, approximately 76% of H37Rv (Fig. 4).

*Growth of M. tuberculosis in mice is reduced but not eliminated by deletion of the regX3 and senX3 genes*

To determine the effect of disrupting the SenX3–RegX3 system on the ability of *M. tuberculosis* to cause progressive infection in mice, we used the wild type and mutant strains to infect mice intravenously. Viable counts in the lungs of the mice 1, 2.5, and 3 months after infection were 10 times lower in those mice infected with the *regX3* or *senX3* mutant compared to the wild type strain (the means of the samples were different compared to the wild type at the 0.01 level using a paired *t* test) (Fig. 5). Counts in the spleens also showed a significant reduction in the mice infected with the mutant. The bacteria were shown to persist for up to 5 months post-injection. Complementation of both mutants with the wild type $senX3^+$–$regX3^+$ alleles resulted in bacterial counts as high as with a wild type experiment, although in the case of the *ΔsenX3* mutant this took longer to achieve, and then only in the lungs. The reason for this difference is unknown but could perhaps be related to the different ratios of *senX3* and *regX3* in the two complemented strains. A previous paper [25] reported that insertion of a transposon into the 5′ end of *regX3* did not lead to attenuation; however, the model of infection was different and perhaps more importantly, the

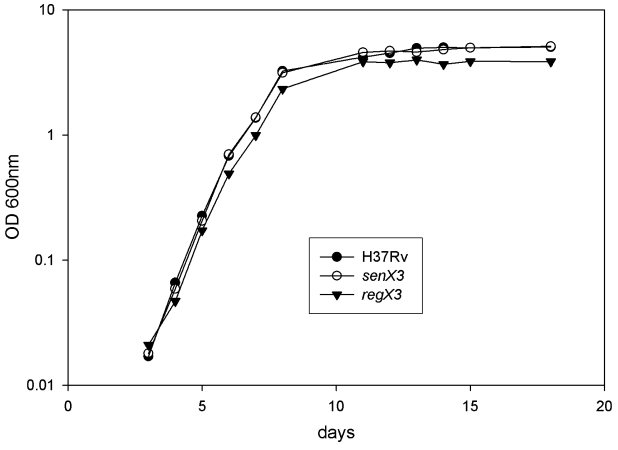


Fig. 4. Growth of the *senX3* and *regX3* mutants and the wild type H37Rv strain in vitro in rolling bottles as described in Materials and methods.

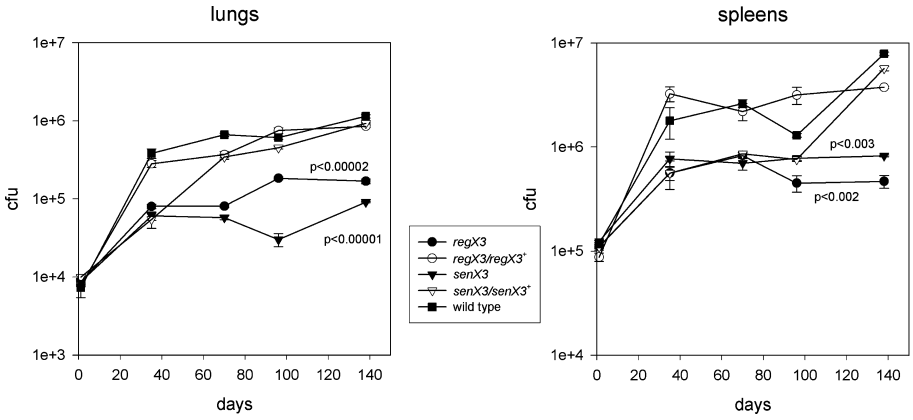


Fig. 5. Growth of the *regX3* and *senX3* null mutants, together with mutants complemented with p*senX3*$^+$–*regX3*$^+$, and the wild type strain H37Rv, in a mouse infection. Error bars indicate the mean (plus or minus the standard error of the mean). The results for each time point were obtained from 3 to 5 infected mice. *p* values indicate that the means of the mutant samples are different from the wild type at that level using a paired Student's *t* test.

complete gene was not deleted so that some residual gene function may have been present. More recently however [26] a strain with a deletion of the 3′ end of *senX3* and most of *regX3* was also reported to have an attenuated phenotype in mice, although since no complementation tests were reported it could not be certain that the *senX3–regX3* mutation was the cause of the attenuated phenotype. This mutant, in contrast to the mutants reported in this paper, also had a growth defect in vitro perhaps caused by the deletion resulting in a hybrid SenX3–RegX3 protein. The results presented in this paper and reproduced many times definitely show that these mutants are attenuated. An intact acquired immune response was not required for this attenuation since growth of the *regX3* mutant in nude mice was still attenuated compared with growth of the wild type (data not shown) despite these mice lacking a thymus and as a consequence being unable to generate mature T lymphocytes and unable to mount most types of immune responses [27].

These experiments demonstrate that an intact SenX3–RegX3 two-component system is required for producing a progressive infection in this model system but that the mutants were still able to persist throughout the course of the infection, and that this attenuation was not due to a general lowering of growth rate as measured in vitro.

## Discussion

PAS (PER-ARNT-SIM) domains are signalling domains that are widely distributed in proteins from members of the Archaea and Bacteria and from fungi, plants, insects, and vertebrates. They function as input modules in proteins that sense oxygen, redox potential, light, and several other stimuli, the specificity in sensing arising, in part, from different cofactors that may be associated with the PAS fold. PAS proteins are always located intracellularly but may monitor the external as well as the internal environment, and in prokaryotes, PAS domains are found almost exclusively in the input domain of sensors of two-component signal transduction systems [28]. The identification of highly conserved sensory regions ($S_1$ and $S_2$ boxes) by Zhulin et al. [14] in a large family of sensor proteins enabled the identification of many well-known sensory proteins as PAS containing proteins that were previously not recognised as members of the PAS domain superfamily. Many of the newly identified PAS containing proteins have been shown to specifically sense oxygen or redox potential and include the Aer protein, an *E. coli* signal transducer that responds to changes in the concentration of oxygen, redox carriers, and carbon sources [29], and FixL from *Rhizobium meliloti* which has been shown to be an oxygen sensing protein [30].

The ability to survive exposure to oxidative stress has long been thought to be important for intracellular pathogens. *M. tuberculosis* is challenged by this type of stress when it is phagocytosed by the macrophage during the initial stages of infection. A dramatic increase in oxidative products is also observed in macrophages that have been activated by interferon-γ as a result of the antigen/antigen-presenting cell/T-cell interactions and the consequent development of an acquired immune response. This gives rise to superoxides and hydrogen peroxide that must be inactivated before they give rise to more toxic reactive oxygen intermediates (ROIs) [31,32]. It is thought that the macrophage utilises this burst of ROIs to cause damage to the bacterial membranes, proteins, and DNA. The bacterium has evolved enzymic systems such as superoxide dismutases, catalases, and hydroperoxidases to detoxify the ROIs, which can also arise from normal bacterial metabolism.

There is good evidence that SenX3 and RegX3 do act as a two-component signal transduction system since phosphotransfer from *senX3* to *regX3* involving conserved histidine and arginine residues has been demonstrated [15]. Since SenX3 contains a PAS-like domain known to be involved in oxygen and redox sensing, and is required for virulence, this two-component system may therefore be required for the protective response to oxidative stress in *M. tuberculosis*. However, a number of results call into question the importance of the oxidative stress response in virulence of *M. tuberculosis*. Curiously, the *oxyR* gene, the central regulator of the peroxide stress response [33,34] and also a regulator of the nitrosative stress response [35], is actually a pseudogene in *M. tuberculosis*, inactivated by multiple mutations [36,37], despite being functional in other mycobacterial species. In mycobacteria lacking *oxyR* it appears that FurA is the dominant regulator of the response to peroxide stress, being a negative regulator of *katG* coding for catalase–peroxidase, but not apparently of *ahpC*, coding for a subunit of alkyl hydroperoxidase reductase that detoxifies organic peroxides and hydrogen peroxide, or *sodA*, the latter coding for superoxide dismutase [38–40]. Moreover, AhpC [37,41–43] has been reported to be produced constitutively in exponentially growing *M. tuberculosis*, its level not responding to oxidative stress [37]. In addition, a null mutant in *ahpC* has recently been reported to be unaffected in a mouse infection model of TB [44]. However, another study [45] using antisense RNA to *ahpC* in *M. bovis* did report markedly reduce virulence. Therefore, it is not clear whether the oxidative stress response is important for intracellular survival. Moreover, since killing of *M. tuberculosis* still occurs in the absence of phagocyte oxidase (phox) [46], interest has been generated in reactive nitrogen intermediates controlling tuberculosis [47], especially peroxynitrite which could be generated by the reaction of nitric oxide and superoxide; this latter could be produced independently of phox by the bacterium's own respiration. If SenX3–RegX3 is a true orthologue of the *E. coli* ArcB–ArcA system, it may therefore be that it is responding to oxidative stress generated internally through intermediary metabolism rather than from that generated by the macrophage.

Our work demonstrates the use of careful structural and sequence analysis to identify important virulence determinants in *M. tuberculosis* as possible orthologues of eukaryotic as well as prokaryotic genes. Three-dimensional structure prediction and modelling enabled us to identify an atypical PAS domain in the sensor region of SenX3 and, as a consequence, to postulate a role for SenX3/RegX3 in oxygen sensing. As suggested previously, broad-spectrum antimicrobials that can block two-component signal transduction systems may be of some therapeutic value [7]. An alternative strategy would be the design of drugs that bind and disrupt the function of the atypical PAS domain in SenX3. Importantly *senX3* and *regX3* null bacteria show dramatic reduction in virulence in mice but persist for up to 5 months post-infection. This suggests that this mutant has the necessary infective characteristics required for an attenuated vaccine. Live vaccines for TB are likely to require the generation of strains with multiple mutations affecting virulence; we contend that mutations in the *senX3–regX3* signal transduction system will be a useful component of such a strain, and their vaccine potential is currently under investigation.

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