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The *senX3–regX3* two-component regulatory system of *Mycobacterium tuberculosis* is required for virulence

Tanya Parish,[1,2] Debbie A. Smith,[2] Gretta Roberts,[1] Joanna Betts[3] and Neil G. Stoker[2,4]

Correspondence
Tanya Parish
t.parish@qmul.ac.uk

[1]Department of Medical Microbiology, Barts and the London, Queen Mary's School of Medicine and Dentistry, Turner Street, London E1 2AD, UK

[2]Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

[3]GlaxoSmithKline, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, UK

[4]Department of Pathology and Infectious Diseases, Royal Veterinary College, Royal College Street, London NW1 0TU, UK

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Two-component regulatory systems have been widely implicated in bacterial virulence. To investigate the role of one such system in *Mycobacterium tuberculosis*, a strain was constructed in which the *senX3–regX3* system was deleted by homologous recombination. The mutant strain (Tame15) showed a growth defect after infection of macrophages and was attenuated in both immunodeficient and immunocompetent mice. Competitive hybridization of total RNA from the wild-type and mutant strains to a whole-genome microarray was used to identify changes in gene expression resulting from the deletion. One operon was highly up-regulated in the mutant, indicating that *regX3* probably has a role as a repressor of this operon. Other genes which were up- or down-regulated were also identified. Many of the genes showing down-regulation are involved in normal growth of the bacterium, indicating that the mutant strain is subject to some type of growth slow-down or stress. Genes showing differential expression were further grouped according to their pattern of gene expression under other stress conditions. From this analysis 50 genes were identified which are the most likely to be controlled by RegX3. Most of these genes are of unknown function and no obvious motifs were found upstream of the genes identified. Thus, it has been demonstrated that the *senX3–regX3* two-component system is involved in the virulence of *M. tuberculosis* and a number of genes controlled by this system have been identified.

INTRODUCTION

Bacteria sense their environment by means of two-component regulatory systems (2CRs) (Hoch, 2000; Smith *et al.*, 2001; Urao *et al.*, 2000). These systems enable organisms to respond to changing environmental conditions by co-ordinated gene regulation. Each system consists of a sensor which responds to a particular stimulus and a regulator which controls the expression of a set of genes. The sensory protein autophosphorylates in response to the stimulus and this phosphate is then transferred to a conserved aspartate residue in the regulatory protein. The regulator is a DNA-binding protein that acts as a transcriptional regulator and can turn sets of genes on or off. Genes that are directly controlled by the same regulator in this fashion are termed the regulon. In addition to direct control, regulatory cascades can occur where one regulator controls the expression of another thus magnifying the number of genes controlled in response to the original signal. 2CRs have been widely implicated in the virulence of pathogenic bacteria, since they can control sets of virulence genes (Dziejman & Mekalanos, 1995). 2CRs have been suggested as potential drug targets as they are not found in higher eukaryotes (Barrett & Hoch, 1998) and attenuated 2CR mutant strains have also been proposed as vaccine candidates (Garcia Vescovi *et al.*, 1996; Groisman & Heffron, 1995). Since each 2CR can control many different genes, it is possible that inactivating one of these systems, either by way of deletion or by use of inhibitors, would be a more effective approach than targeting an individual enzyme and would be less likely to lead to the development of resistance problems.

Mycobacterium tuberculosis is a sophisticated pathogen that can persist in the human host for many years. The bacteria

Abbreviations: BMDM, bone-marrow-derived macrophage; 2CR, two-component regulatory system.

are exposed to many different conditions during the infection and disease process and must have well-tuned mechanisms to respond to any given environment. For example, the bacteria can be found growing both intracellularly (in macrophages) or extracellularly (in the granuloma). Since the function of 2CRs is to adapt to different external conditions, they are likely to play an important role in the ability of *M. tuberculosis* to sense and respond to different host environments.

The current TB vaccine (*Mycobacterium bovis* BCG), a live attenuated strain, is not ideal due to its variable efficacy. Several approaches are being taken to try to improve the vaccine, of which one is to rationally attenuate *M. tuberculosis*. A genetic knock-out in one of the 2CRs may be a valuable component of such a vaccine strain, as many relevant genes may be affected at the same time. In addition, identifying genes that are regulated by these systems may lead to the identification of new virulence factors. This will increase our knowledge of pathogenesis and may allow for the development of novel interventions.

M. tuberculosis has 11 2CRs, as identified from the genome sequence (Cole *et al.*, 1998), and several of these have been implicated in virulence. For example, PhoP and Prr mutants are attenuated (Ewann *et al.*, 2002; Perez *et al.*, 2001), whereas DevR, TcrXY, TrcS and KdpDE mutants are hypervirulent (Parish *et al.*, 2003), showing that these systems do play an important role during infection. The *senX3–regX3* system, originally identified by degenerate PCR, was the first reported example of an *M. tuberculosis* 2CR (Wren *et al.*, 1992). The two genes are separated by a small intergenic region which contains three repeats of a MIRU (mycobacterial interspersed repeat unit), although they are very likely to be co-transcribed. Phosphorylation of the regulator by the sensor has been demonstrated and there is some evidence that the system is auto-regulated with RegX3 binding to its own promoter (Himpens *et al.*, 2000). However, the genes that this system controls have not been identified. We have investigated the role of this system in virulence and used a mutant strain lacking a functional *senX3–regX3* system in order to characterize the regulon.

METHODS

Strains and growth of bacteria. Wild-type *M. tuberculosis* H37Rv (ATCC 25618) and the *senX3–regX3* deletion strain (Tame15) were grown at 37 °C in Middlebrook 7H9 medium (Difco) supplemented with 10 % (v/v) OADC (Becton Dickinson) and 0·05 % (w/v) Tween 80, or on Middlebrook 7H10 agar (Difco) supplemented with 10 % (v/v) OADC. Hygromycin was used at 100 μg ml^{-1}, kanamycin at 20 μg ml^{-1}, streptomycin at 20 μg ml^{-1}, X-Gal at 50 μg ml^{-1} and sucrose at 2 % (w/v) where appropriate.

Construction and confirmation of the *senX3* deletion strain. The *senX3–regX3* deletion was constructed using previously published methods (Parish & Stoker, 2000). Plasmids used in this study are described in Table 1. A deletion delivery construct (pSOUP25) was made using the pNIL and pGOAL series vectors (maps available on request) (Parish & Stoker, 2000). The deletion constructed encompassed the 3′ end of the *senX3* gene, the intergenic region and the 5′ end of the *regX3* gene (Fig. 1). Mutants were constructed using a two-step strategy as described previously. Briefly, 1–5 μg of vector DNA was pre-treated with UV to stimulate homologous recombination and used to electroporate *M. tuberculosis*. Single cross-over strains were selected on agar containing hygromycin, kanamycin and X-Gal. An individual colony was streaked out onto agar (without antibiotics) to allow the second cross-over to occur. Cells were resuspended in media and serial dilutions were plated onto X-Gal and sucrose. Sucrose-resistant, white colonies were tested for kanamycin sensitivity and analysed by PCR and Southern hybridization. PCR primers regP1 (5′-GGTAATTGTTTGAGATCC-CAC-3′) and regP3 (5′-GTCCGCTAGCCCTCGAGTTTG-3′) were used to PCR-amplify the whole operon to distinguish strains carrying the wild-type (2·3 kb) from the deletion allele (1·4 kb) (Fig. 2). The PCR product from the deletion strain was sequence-verified. Genomic DNA was prepared according to the method of Belisle & Sonnenberg (1998). Southern hybridization was carried out using the AlkPhos Direct kit (Amersham) according to the manufacturer's instructions in order to confirm the expected genotype (Fig. 2).

***In vitro* growth curve.** Strains were inoculated into twenty 50 ml capacity tubes each containing 10 ml of liquid media to a theoretical OD_{600} value of 0·05. Cultures were incubated standing at 37 °C and growth was monitored by measuring the OD_{600} value. A fresh tube was used for each time point.

Infection assays. Viable stocks of wild-type *M. tuberculosis* and mutants were grown in 10 ml of liquid medium until an OD_{600} value of between 0·5 and 1·0 was reached. Bacteria were washed once in Dulbecco's PBS (Sigma) and resuspended in 5 ml sterile PBS. Aliquots were used fresh for the THP1 infections or stored at −70 °C until use. At the time of inoculation, serial dilutions were plated to determine the input c.f.u. value.

Table 1. Plasmids used in this study

Name	Description	Source
p2NIL	Cloning vector, *oriE*, *kan*	Parish & Stoker (2000)
pGOAL19	*hyg*, P_{hsp60}–*sacB*, P_{Ag85a}–*lacZ* marker gene cassette, *amp*, *oriE*	Parish & Stoker (2000)
pSOUP20	4·3 kb *Bgl*II–*Bam*HI fragment with *senX3–regX3* in p2NIL	This study
pSOUP21	0·9 kbp deletion of pSOUP20	This study
pSOUP25	pSOUP21 plus *hyg*, P_{hsp60}–*sacB*, P_{Ag85a}–*lacZ* cassette from pGOAL19 (final delivery vector)	This study
pSM128	Promoter-probe vector, *lacZ* reporter gene, *Sm*	Dussurget *et al.* (1999)
pIKL-R1	Upstream region of *senX3–regX3* operon in pSM128, *Sm*	This study

Macrophage infection assays

The THP1 macrophage-like human cell line and bone-marrow-derived macrophages (BMDMs) were used for *in vitro* assays. Macrophage viability over the assay time was typically greater than 95 %. Antibiotics were not added to the cells, since *M. tuberculosis* does not replicate in the medium in this assay.

THP1 infection. THP1 cells were maintained in culture, treated with phorbol 12-myristate 13-acetate to induce differentiation, washed and then infected as described by Lukey (2001). Extracellular bacteria were removed by washing several times. Determination of the initial inoculum was assessed by plating serial dilutions, and the number of intracellular bacteria was monitored over 7 days.

BMDMs. BMDMs from BALB/c mice were isolated and infected in the absence of antibiotics as described previously (Smith *et al.*, 2001). Macrophage monolayers were pre-stimulated with IFNγ (Gibco) at a concentration of 200 units ml^{-1} for 4 h prior to infection. Cells were infected for 4 h and washed six times in warm tissue culture medium to remove extracellular bacteria. The infection dose was assayed independently by plating the inoculum. The number of viable mycobacteria was assessed by lysis of the macrophage monolayer with 1 ml sterile distilled water containing 0·1 % Triton X-100 per well, followed by plating serial dilutions.

Infection of mice and tissue analysis. Mice were infected with 1×10^6 viable mycobacteria in 200 µl pyrogen-free saline via a lateral tail vein. Where appropriate, infected mice were killed by cervical dislocation in accordance with humane end point protocols under the Animals Scientific Procedures Act, 1986 (UK). Median survival times were calculated for each group and statistical analysis was performed using Kaplan–Meier plots and Log Rank tests of survival. For tissue analysis, lungs, livers and spleens were collected aseptically and passed through a 100 micron pore-size sieve (Falcon) in 7H9 medium containing 0·05 % (w/v) Tween 80. Serial 10-fold dilutions were plated and c.f.u. were counted after 4 weeks. Statistical analysis was performed using Student's *t*-test.

Microarray analysis. Wild-type and mutant *M. tuberculosis* were grown in 100 ml media in roller bottles to late-exponential phase (7 days). Cells were harvested by centrifugation and RNA was prepared according to the method of Movahedzadeh *et al.* (2001). Fluorescently labelled cDNA was prepared from total RNA by direct incorporation of fluorescent nucleotide analogues during a first-strand reverse transcription (RT) reaction as described previously (Betts *et al.*, 2002). Wild-type RNA was labelled with Cy3-dCTP and mutant RNA was labelled with Cy5-dCTP, and they were compared directly by competitive hybridization. DNA microarrays used consisted of 3649 PCR-amplified ORF-specific DNA fragments, representing 93 % of the predicted 3924 *M. tuberculosis* H37Rv ORFs, and hybridizations were performed as described previously (Betts *et al.*, 2002). Slides were scanned using a ScanArray 3000 instrument (GSI Lumonics) and the resulting images were analysed using GENEPIX PRO 3.0 software (Axon Instruments). RNA was isolated from three separate cultures and duplicate hybridizations were carried out for each, making a total of six hybridizations. Data from GENEPIX were analysed in GENESPRING (Silicon Genetics). Data points were excluded from the analysis if the spots were flagged as absent or marginal by GENEPIX. For each slide, the normalization was as follows: each gene's measured intensity (mutant) was divided by the control channel value (wild-type) in each sample to give the ratio of expression; when the control channel value was below 10·0 the datum point was considered bad; the 50th percentile of all measurements was used as a positive control for each sample; each measurement for each gene was divided by this synthetic positive control (assuming that this was at least 0·01); normalized values below 0 were set to 0. Genes were defined as being differentially regulated where there was a greater than twofold change in at least four of the hybridizations and where $P < 0{\cdot}05$ by Student's *t*-test. The *t*-test was conducted on the six experiments as a group. Data from published array experiments were imported into GENESPRING. Cluster analysis was then performed to group the genes depending upon their expression pattern using GENESPRING.

Promoter activity assays. The promoter region of the *senX3–regX3* operon was amplified using primers regP1 and regP2 (5′-CAGCGCCGAGAACACAGTCAC-3′) and cloned into pGEM-EasyT vector (Promega). The promoter region was subcloned as a blunt-ended fragment in the forward orientation into the *Sca*I site of the promoter-probe shuttle vector pSM128 to make plasmid pIKL-R1. The plasmid was electroporated into wild-type and Tame15 strains, and transformants were selected on streptomycin. Transformants were grown in 10 ml liquid medium to late-exponential phase before assaying for β-galactosidase activity as described previously (Parish *et al.*, 2001). Three independent transformants were each assayed in duplicate.

RESULTS AND DISCUSSION

Construction of the *senX3–regX3* mutant, Tame15

We have previously examined the role of 2CRs in the virulence of *M. tuberculosis* by constructing several deletion strains. Of five deletion strains studied, four (*devR*Δ, *tcrXY*Δ, *trcS*Δ and *kdpDE*Δ) were found to be significantly more virulent in a mouse model of infection (Parish *et al.*, 2003). We extended these studies to look at another system (*senX3–regX3*) in more detail. We constructed a mutant strain of *M. tuberculosis* containing a deletion of the *senX3–regX3* system by homologous recombination. A strain containing a 0·9 kbp deletion encompassing the 3′ end of *senX3*, the intergenic region and the majority of *regX3* was constructed using a two-step homologous recombination method (Parish & Stoker, 2000). The deletion is shown in Fig. 1. The replacement of the wild-type gene by the deleted version was confirmed by both PCR and Southern analysis (Fig. 2). Motif analysis shows that the sensor protein has a histidine kinase domain located near the centre of the protein and a phospho-acceptor domain at the C-terminal end. The regulator has a response regulator phospho-receiver domain in the N-terminal region and the DNA-binding effector domain in the C-terminal end. The deletion we constructed completely removes the phospho-receiver domain and the majority of the DNA-binding domain from RegX3 whilst both the sensor protein domains are left intact.

Growth characteristics

We first looked at the growth characteristics of the mutant in axenic culture (Fig. 3). The mutant behaved erratically, in that on some occasions growth resembled the wild-type strain (Fig. 3a) whilst on other occasions there seemed to be a clear defect (Fig. 3b). Small differences in the inoculum

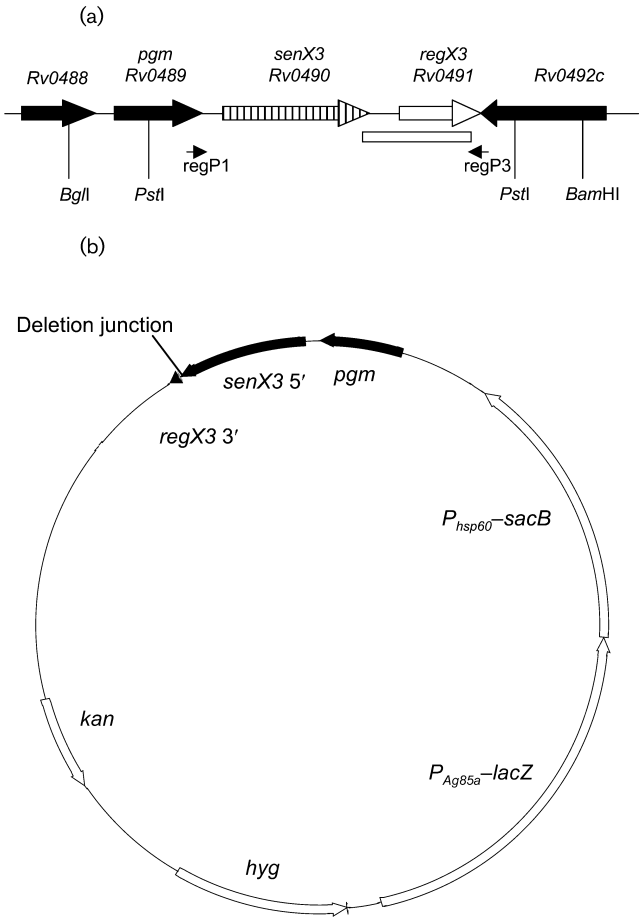


Fig. 1. Construction of the *senX3–regX3* deletion mutant strain. (a) Arrangement of genes in the *M. tuberculosis* chromosome showing the regions used in the construction of pSOUP25. Open arrow, response regulator; hatched arrow, sensor; solid arrows, other genes; open box, region deleted in the mutant. Relevant restriction sites are shown. regP1 and regP3 were the PCR primers used for characterizing strains (see Fig. 2). (b) pSOUP25 delivery vector used for mutagenesis. The 4·3 kb *Bgl*II–*Bam*HI *M. tuberculosis* fragment indicated in (a) was cloned into p2NIL and a 0·9 kbp deletion was subsequently made. The marker gene cassette from pGOAL19 was added to make the final delivery vector. *kan*, kanamycin resistance gene; *hyg*, hygromycin resistance gene; P_{Ag85a}–*lacZ*, β-galactosidase driven by the mycobacterial antigen 85 A promoter; P_{hsp60}–*sacB*, sucrose sensitivity gene driven by the mycobacterial Hsp60 promoter.

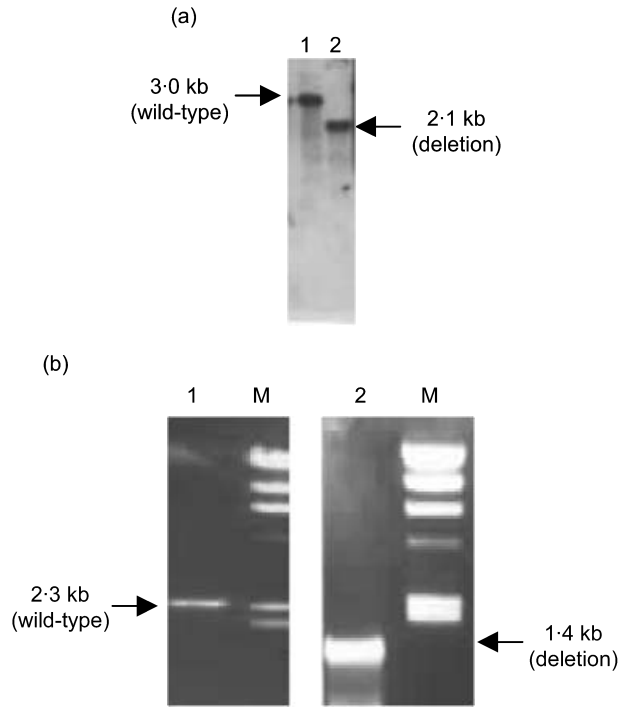


Fig. 2. Genetic analysis of the *senX3–regX3* mutant. (a) Southern analysis. Genomic DNA was digested with *Pst*I and then hybridized to the *senX3–regX3* region probe. The wild-type (3·0 kbp) and deletion bands (2·1 kbp) are indicated. (b) PCR analysis. The PCR primers regP1 and regP3 were used to amplify the region from the wild-type and mutant strains. The expected sizes for wild-type (2·3 kbp) and deletion (1·4 kbp) bands are indicated. Lanes: 1, wild-type; 2, Tame15 deletion strain; M, lambda *Hin*dIII markers.

with respect to growth phase, number of bacteria or other factors may be responsible for the inconsistent pattern of growth. Since *M. tuberculosis* is such a slow-growing organism, very small differences in the initial inoculum could be magnified during prolonged growth. The failure of the mutant to grow to the same optical density value as the wild-type was noted on numerous occasions but was not perfectly reproducible. Thus, we concluded that the mutant had a subtle growth defect which we could not quantify precisely.

Macrophage assays

To determine whether the mutant was attenuated in terms of its ability to survive and grow intracellularly, we looked at growth within two different cell types. The growth of the mutant was assayed in the macrophage-like THP1 cell line (Fig. 4a) over several days. As can be seen in Fig. 4(a), the mutant showed a high degree of attenuation at both low and high m.o.i. In the low m.o.i. infection, the mutant was killed by the macrophages and completely cleared by day 7, whereas the wild-type remained viable and increased in number. In the high m.o.i. infection, the mutant survived and replicated but not to the same extent as the wild-type. We then looked at growth within IFNγ-primed murine BMDMs over 3 days (Fig. 4b). The mutant showed attenuation in this system as well. There was a significantly higher rate of killing, which was apparent at the earliest time point post-infection (24 h, $P<0{\cdot}0007$). Thus, the mutant showed a clearly reduced ability to survive within a macrophage cell line and activated primary BMDMs.

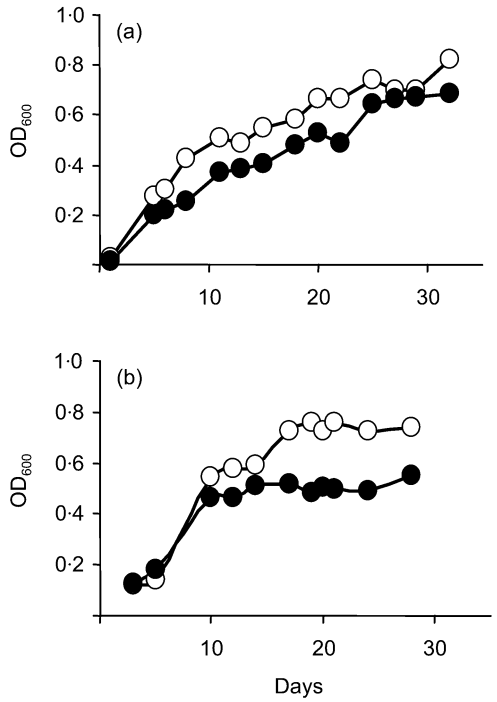


Fig. 3. Growth characteristics of Tame15 in axenic culture. Two growth curves are shown for the mutant and deletion strains representing the two different types of growth observed. (a) Normal growth; (b) restricted growth. ○, Wild-type; ●, Tame15.

Virulence in two mouse models

Since the mutant showed attenuation in both *in vitro* models used, we used two different mouse models of infection to establish whether the mutation in the *senX3–regX3* system also altered the virulence of *M. tuberculosis in vivo*. To look at infection in the absence of acquired immunity, SCID mice were infected intravenously and monitored for survival (Fig. 5). Infection with wild-type *M. tuberculosis* H37Rv led to death with a median survival time (MST) of 40·5 days. The mutant strain was significantly less virulent with a MST of 47 days ($P<0·0005$). We then looked at the kinetics of bacterial growth in the immunocompetent host using DBA/2 mice. Bacterial loads in the organs were measured on days 15, 30 and 59 (Fig. 6). The bacterial loads in the livers of mice infected with the mutant were significantly lower on days 15 and 30, and by day 59 a reduction in numbers was also apparent in the lungs. Thus, the mutant had mildly reduced virulence in immunocompetent mice in the early stages of infection.

A *regX3* transposon mutant of strain Mt103 has previously been shown not to be attenuated using a low dose (10^3 bacteria) aerosol infection model of C57BL/6J mice, as assessed by bacterial loads in the lungs over 40 days (Ewann *et al.*, 2002). The difference between these results and ours may be due to differences in the model, i.e. the route of infection, infection dose and mouse strain. For example, we used DBA mice which are more susceptible to infection than C57BL/6J mice. Alternatively, it may reflect a real difference in the mutants themselves. The transposon mutant is an insertion into the 5′ end of *regX3* and may not completely abrogate RegX3 function, whereas our mutant has the majority of the gene and both domains deleted. Tame15 also has the 3′ end of *senX3* deleted and although it seems unlikely that this would abrogate SenX3 function, it cannot be ruled out. There are precedents for a sensory protein interacting with more than one regulator and if this were the case then our mutant may have a different phenotype due to the additional absence of SenX3 function. Alternatively, it may be the fact that the transposon mutant was constructed in a different genetic background (strain Mt103 as opposed to H37Rv). Tame15 was attenuated in all four systems tested, so we are confident that this is a real phenotype. The *in vivo* results are also consistent with the defect in axenic growth.

The reason for the attenuation of Tame15 is not yet known. It is possible that the attenuation may result from a general decrease in the growth rate; however, this is true of many strains, e.g. auxotrophic strains are attenuated because their growth is severely restricted by amino acid availability (Smith *et al.*, 2001). Nevertheless, Tame15 is an attenuated strain, whatever the root cause. The difference seen in the SCID mouse model was not due to small variations in inoculum size, since the experiment was conducted twice with similar results (inocula sizes 1×10^6 vs 2×10^6 and 4×10^6 vs 2×10^6, respectively).

Promoter activity

To look at promoter activity for the *senX3–regX3* system in the wild-type and mutant strain we cloned the upstream promoter region into the integrating vector pSM128 (Dussurget *et al.*, 1999) (Fig. 7). Using the β-galactosidase reporter gene, we were able to assess promoter activity under growth in aerobic cultures. Promoter activity was low in both strains, but unexpectedly the promoter activity was 2·8-fold increased in the mutant strain (wild-type, $2\pm0·4$ units; Tame15, $5·6\pm0·7$ units; $P<0·00001$).

There is some indirect evidence for auto-regulation of RegX3. Himpens *et al.* (2000) showed that there was twofold higher promoter activity of the *M. tuberculosis* promoter in *Mycobacterium smegmatis* when the strain also carried the *M. tuberculosis senX3–regX3* operon. In addition, they showed that RegX3 binds to its own promoter in the absence of phosphorylation. In contrast, we see that promoter activity is actually higher in the mutant strain. We can explain this apparent discrepancy if RegX3 actually represses promoter activity in the unphosphorylated state by physically blocking RNA polymerase access to the promoter. In the phosphorylated state, it would be expected to undergo a conformational change which would lead

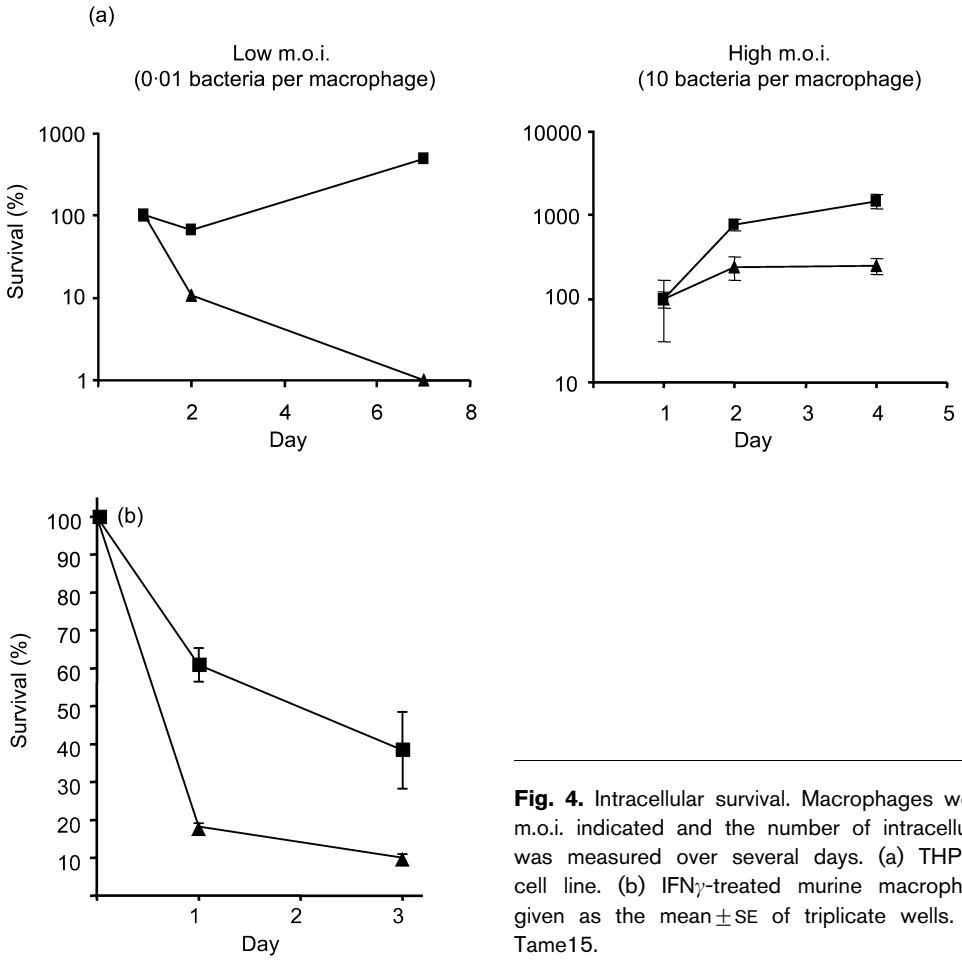


Fig. 4. Intracellular survival. Macrophages were infected at the m.o.i. indicated and the number of intracellular viable bacteria was measured over several days. (a) THP1 macrophage-like cell line. (b) IFNγ-treated murine macrophages. Results are given as the mean $\pm$ SE of triplicate wells. ■, Wild-type; ▲, Tame15.

to promoter activation (possibly by recruiting RNA polymerase). In our mutant in the absence of RegX3, promoter activity would be slightly higher as there is no unphosphorylated RegX3 bound and therefore no steric hindrance to RNA polymerase. Alternatively, the auto-regulation seen in *M. smegmatis* may not occur in *M. tuberculosis*.

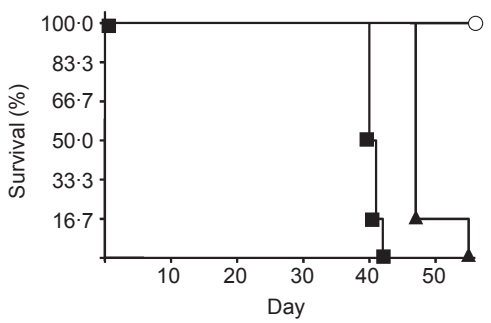


Fig. 5. Virulence of Tame15 in SCID mice. Survival of SCID mice after infection with 1×10^6 bacteria. Each group contained six mice and results are representative of two separate experiments. ■, Wild-type; ▲, Tame15; ○, control (PBS).

Analysis of global gene expression

Two-component systems generally function as global regulators of gene expression in response to environmental conditions. Each system controls a set of genes, termed the regulon, in response to a particular signal. To identify potential members of the *senX3–regX3* regulon, we compared global gene expression in the mutant with that of the wild-type strain. The reporter assays confirmed that the system is expressed under aerobically grown conditions, so we would expect to see changes in expression of genes controlled by this 2CR under these conditions. Therefore, RNA was prepared from both strains grown in roller bottles (aerobic) and competitively hybridized to a whole-genome microarray. Genes showing a significant difference in expression in the mutant were identified and are given in Table 2. As expected from the deletion constructed, *regX3* transcripts were significantly reduced; however, the *senX3* transcript was unchanged (expression ratio not significantly different from 1). Thus, the change in *senX3* promoter activity observed in the reporter gene was not reflected in the array data. This may be because of the low absolute activity of the promoter, so that the twofold increase was below the limit of detection of the microarray. We confirmed the absence of *regX3* mRNA by

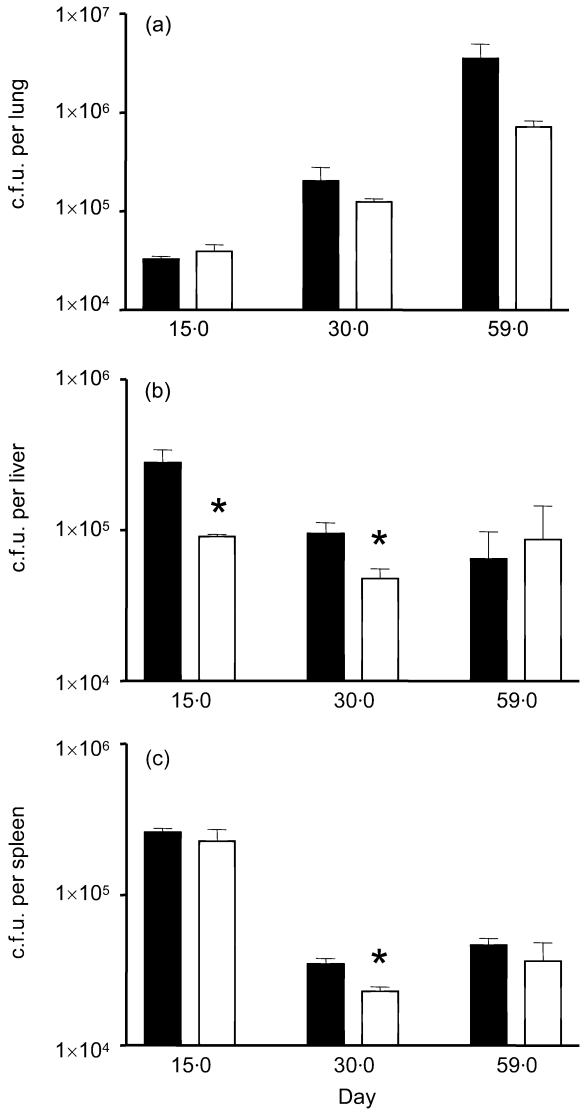


Fig. 6. Virulence of Tame15 in DBA mice. Mice were infected intravenously with 1×10^6 wild-type or mutant bacteria and organ loads were measured. The results represent means ± SE for three mice per group with significance measured using Student's *t*-test. An asterisk indicates where $P < 0{\cdot}05$. Solid bars, wild-type; open bars, Tame15. (a) Lung; (b) liver; (c) spleen.

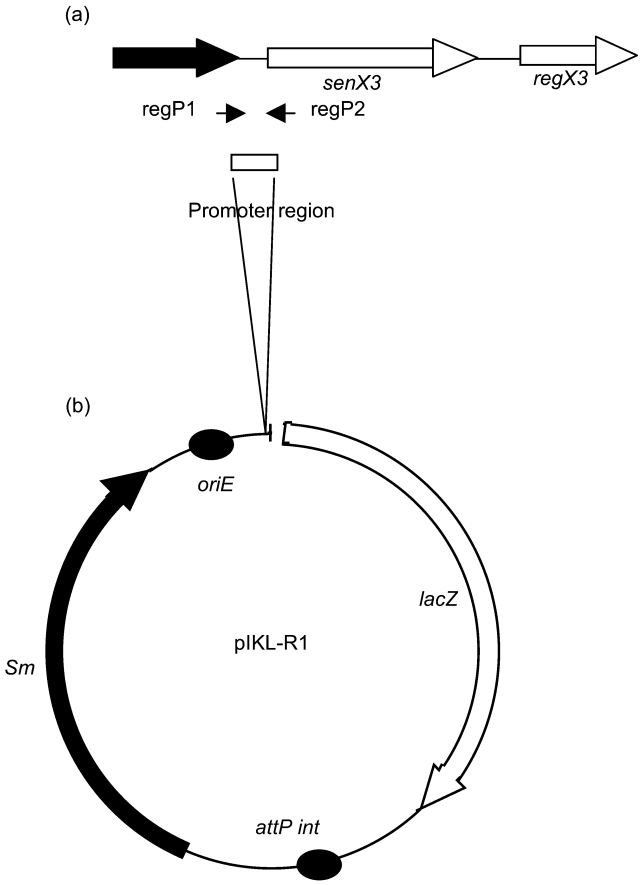


Fig. 7. Promoter activity analysis. (a) The upstream promoter region of the *senX3–regX3* operon was amplified using the primers regP1 and regP2 and cloned into the shuttle vector pSM128 upstream of the *lacZ* reporter gene making pIKL-R1 (b). pIKL-R1 was transformed into wild-type and Tame15 strains.

quantitative RT-PCR and found that there was 100-fold less *regX3* mRNA in the mutant strain (data not shown).

In total, 30 genes were up-regulated and 68 genes were down-regulated. From these results it is not possible to determine which effects are direct and which are indirect. However, in yeast, a survey of 106 regulatory proteins showed that they interacted with between 0 and 180 promoter regions with a mean of 38, so our tally of genes is well within the expected range (Lee *et al.*, 2002).

Differential gene expression

At this stage, it is not possible to determine which genes are directly controlled at the transcriptional level by RegX3 and which genes may be indirectly controlled, for example, via other regulators. However, we can say that all of the genes whose expression changes must rely on the *senX3–regX3* system in some way for normal expression. If RegX3 directly controls the transcription of these genes, then it must be a negative regulator of up-regulated genes, i.e. it represses the expression of such genes. In contrast, for those genes that are down-regulated in the mutant, RegX3 would act as a positive regulator (inducer). More genes were expressed at a lower level in the mutant than were de-repressed, indicating that a larger number of genes rely on the *senX3–regX3* system for normal expression. The possibility of regulatory cascades is raised by the array data since there are four potential transcriptional regulators whose expression changes, two going up in the mutant (*Rv1990c* and *Rv2669*) and two going down (*Rv2488c*

Table 2. Whole-genome microarray analysis of gene expression in Tame15

Genes that were identified as being differentially expressed in the *senX3–regX3* mutant are listed. The *Rv* numbers from Cole *et al.* (1998) are given for reference, together with any gene designation, the functional classification assigned, the mean fold change in expression, the *P* value and the predicted function.

Class	Functional classification	ORF	Gene	Ratio	*P* value	Product
	Up-regulated genes					
1.A	Degradation	*Rv2780*	*ald*	3·19	0·003	L-Alanine dehydrogenase
1.B	Energy metabolism	*Rv1622c*	*cydB*	2·90	0·02	Cytochrome *d* ubiquinol oxidase subunit II
		Rv1131	*gltA1*	4·00	0·04	Citrate synthase 3
I.G	Biosynthesis of cofactors, prosthetic groups and carriers	*Rv1470*	*trxA*	2·05	0·03	Thioredoxin
I.I	Polyketide and non-ribosomal peptide synthesis	*Rv0101*	*nrp*	12·4	0·003	Non-ribosomal peptide synthase
I.J	Broad regulatory functions	*Rv1990c*		2·24	0·04	Putative transcriptional regulator
		Rv2669		2·52	0·0003	Putative transcriptional regulator
III.A	Transport/binding proteins	*Rv0103c*	*ctpB*	2·44	0·005	Cation transport ATPase
III.F	Detoxification	*Rv3846*	*sodA*	2·14	0·0007	Superoxide dismutase
IV.B	IS elements, repeated sequences and phages	*Rv2424c*		2·05	0·007	Transposase
		Rv2647		2·57	0·04	phiRV2 phage-related protein
		Rv3750c		2·36	0·0003	Excisionase
IV.C	PE and PPE families	*Rv1089*	*PE10*	2·03	0·04	PE family protein
		Rv0096	*PPE1*	12·4	0·003	PPE family protein
IV.E	Bacteriocin-like proteins	*Rv3660c*		2·07	0·04	
V	Conserved hypothetical proteins	*Rv0097*		17·1	0·0003	
		Rv0516c		2·06	0·05	
		Rv0807		2·27	0·04	
		Rv1284		3·17	0·008	
		Rv1996		2·14	0·05	
		Rv2626c		2·15	0·04	
		Rv2638		2·13	0·009	
VI	Unknowns	*Rv2668*		2·51	0·0008	
		Rv2337c		3·94	0·02	
		Rv2633c		2·10	0·00003	
		Rv3572		2·07	0·0003	
		Rv0098		15·9	0·009	
		Rv0100		5·24	0·03	
		Rv3749c		2·84	0·002	
		Rv3890c		2·08	0·02	
	Down-regulated genes					
I.A	Degradation	*Rv0271c*	*fadE6*	0·43	0·03	Acyl-CoA dehydrogenase
		Rv0974c	*accD2*	0·30	0·006	Acetyl/propionyl-CoA carboxylase
		Rv1346	*fadE14*	0·38	0·0002	Acyl-CoA dehydrogenase
		Rv3140	*fadE23*	0·48	0·02	Acyl-CoA dehydrogenase
I.B	Energy metabolism	*Rv0183*		0·38	0·005	Probable oxidoreductase
		Rv1714		0·28	0·01	Probable oxidoreductase
		Rv1812c		0·46	0·005	Probable dehydrogenase
I.D	Amino acid biosynthesis	*Rv2988c*	*leuC*	0·30	0·003	3-Isopropylmalate dehydratase
I.F	Purines, pyrimidines, nucleosides and nucleotides	*Rv0803*	*purL*	0·49	0·02	Phosphoribosylformylglycinamidine synthase II
I.G	Biosynthesis of cofactors, prosthetic groups, carriers	*Rv1355c*	*moeY*	0·39	0·03	Weak similarity to *Escherichia coli* MoeB
I.H	Lipid biosynthesis	*Rv2244*	*acpM*	0·45	0·02	Acyl carrier
		Rv3229c	*desA3*	0·42	0·01	Acyl-(ACP) desaturase
		Rv0129c	*fbpC2*	0·49	0·0002	Antigen 85C, mycolyl transferase

Table 2. cont.

Class	Functional classification	ORF	Gene	Ratio	*P* value	Product
I.J	Broad regulatory functions	*Rv2488c*		0·49	0·01	Transcriptional regulator
		Rv2308		0·34	0·007	Putative transcriptional regulator
		Rv0491	*regX3*	0·40	0·02	Two-component response regulator
II.A.1	Ribosomal protein synthesis and modification	*Rv0651*	*rplJ*	0·48	0·0007	50S ribosomal protein L10
		Rv0705	*rpsS*	0·49	0·003	30S ribosomal protein S19
		Rv0706	*rplV*	0·44	0·0001	50S ribosomal protein L22
		Rv0707	*rpsC*	0·46	3×10^{-7}	30S ribosomal protein S3
		Rv0710	*rpsQ*	0·49	0·02	30S ribosomal protein S17
II.A.5	DNA replication, repair, recombination and restriction/modification	*Rv0058*	*dnaB*	0·48	0·02	DNA helicase (contains intein)
		Rv2594c	*ruvC*	0·38	0·0002	Holliday junction resolvase
		Rv3644c		0·45	0·009	Similar in N-terminal to DNA polymerase III
		Rv3711c	*dnaQ*	0·48	0·0003	DNA polymerase III (epsilon) chain
II.A.7	RNA synthesis, modification and DNA transcription	*Rv2783c*	*gpsI*	0·50	0·01	pppGpp synthase
II.B	Degradation of macromolecules	*Rv3419c*	*gcp*	0·47	0·01	Glycoprotease
II.C	Cell envelope	*Rv1252c*	*lprE*	0·48	0·03	Lipoprotein
		Rv1518		0·24	0·01	Involved in exopolysaccharide synthesis
		Rv0867c		0·35	0·004	Probable exported protein
		Rv1433		0·49	0·01	Possible membrane protein
		Rv1457c		0·47	0·02	Probable membrane protein
III.A	Transport/binding proteins	*Rv2040c*		0·42	0·004	Probable sugar transporter
		Rv2835c	*ugpA*	0·37	0·03	*sn*-Glycerol-3-phosphate permease
III.B	Chaperones/heat shock	*Rv0351*	*grpE*	0·41	0·0001	Stimulates DnaK ATPase activity
III.F	Detoxification	*Rv2428*	*ahpC*	0·48	0·008	Alkyl hydroperoxide reductase
		Rv2429	*ahpD*	0·27	0·0002	Member of AhpC/TSA family
IV.A	Virulence	*Rv3100c*	*smpB*	0·50	0·02	Probable small protein b
IV.B	IS elements, repeated sequences and phages	*Rv1199c*	*IS*	0·41	0·001	
		Rv3023c		0·42	0·001	
		Rv1148c		0·48	0·02	REP family protein
		Rv2650c		0·44	0·03	phiRV2 phage-related protein
IV.C	PE and PPE families	*Rv0916c*	*PE7*	0·13	0·001	
		Rv1195	*PE13*	0·34	3×10^{-6}	
		Rv3020c	*PE28*	0·46	0·01	
		Rv2430c	*PPE41*	0·42	0·00001	
IV.I	Miscellaneous phosphatases, lyases and hydrolases	*Rv3310*		0·47	0·03	Probable acid phosphatase
V	Conserved hypothetical proteins	*Rv1713*	*engA*	0·45	0·01	
		Rv0282		0·42	0·00007	
		Rv0312		0·50	0·02	
		Rv0647c		0·35	0·007	
		Rv0839		0·36	0·007	
		Rv1330c		0·19	0·02	
		Rv1592c		0·30	0·004	
		Rv1942c		0·24	0·008	
		Rv2757c		0·41	0·01	
		Rv2760c		0·13	0·0005	
		Rv2866		0·47	0·02	
		Rv3353c		0·41	0·05	
		Rv3586		0·48	0·0007	
VI	Unknowns	*Rv2687c*		0·46	0·01	
		Rv3587c		0·45	0·00001	
		Rv0616c		0·50	0·05	
		Rv1116		0·33	0·04	

Table 2. cont.

Class	Functional classification	ORF	Gene	Ratio	*P* value	Product
		Rv2274c		0·42	0·0003	
		Rv3861		0·49	0·01	
		Rv0430		0·49	0·0002	
		Rv1891		0·43	0·02	

and *Rv2308*). In addition, two other up-regulated genes show some similarity to anti-anti-sigma factors (*Rv2638*, *Rv0516c*). In yeast, several different types of networks of regulatory proteins have been identified (Lee *et al.*, 2002) and it is likely that similar networks exist in bacteria.

Down-regulated genes

Most genes that were significantly down-regulated decreased by a factor of two- to threefold. More genes appear to be repressed than induced in the mutant and these fall into several categories. Many of the genes are involved in basic macromolecule biosynthesis, particularly DNA and RNA synthesis. We have previously seen that deletion of a 2CR can have an indirect effect on gene expression. With TrcS the cells appeared to be stressed and several genes were differentially expressed in response to the stress rather than as a direct result of the regulator deletion (Wernisch *et al.*, 2003). The data for this strain look as if a similar stress response is occurring.

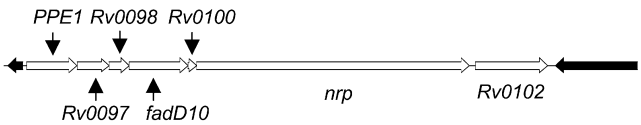
Several ribosomal proteins are down-regulated as are genes involved in DNA replication, repair and recombination (*dnaB*, *ruvC*, *dnaQ*, *Rv3644c*) as well as insertion elements. This would seem to indicate that the mutant would have a slower growth rate than the wild-type since it is less capable of synthesizing new DNA, RNA and protein. This is consistent with the growth phenotype observed earlier.

A number of genes involved in fatty acid degradation are repressed (*fadE6*, *accD2*, *fadE14*, *fadE23*) as are several probable oxidoreductases and dehydrogenases (*Rv0183*, *Rv1714*, *Rv1812c*). There are also a number of genes involved in cell-wall biosynthesis, including lipid biosynthesis (*acpM*, *desA3*, *fbpC2*) and the cell envelope (membrane and exported proteins; *lprE*, *Rv0867c*, *Rv1433*, *Rv1457c*). The alkyl-hydroperoxidases *ahpC* and *ahpD* are both down-regulated, whereas superoxide dismutase (*sodA*) is up-regulated, suggesting that there is a change in the type of oxidative stress the cells are facing internally, rather than a general increase in stress. Several members of the PE/PPE family are down-regulated as are a large number of conserved hypothetical and unknown proteins.

Up-regulated genes

Most genes that were significantly up-regulated increased by a factor of two- to threefold. However, the expression of one operon (*Rv0096* to *Rv0101*) was highly elevated. The level of induction in the mutant was five- to 17-fold over that in the wild-type (Fig. 8). Since the *Rv0096* operon was so highly induced, it seems likely that this operon is controlled directly by the RegX3 regulator rather than via an indirect effect, due to, for example, slowed growth rate or increased intracellular stress levels. The operon looks to consist of several genes comprising at least *Rv0096* to *Rv0100* and possibly *Rv0101* and *Rv0102* as well (Fig. 8). Four of these genes are significantly up-regulated in the mutant. *Rv0096* encodes a member of the PE/PPE family whose function is unclear, although these proteins have been proposed to be involved in antigenic variation (Banu *et al.*, 2002), and other members of this family have been shown to be up-regulated in the frog model of mycobacterial-induced granuloma formation (Ramakrishnan *et al.*, 2000), suggesting a role in pathogenicity. *Rv0097* encodes a possible oxidoreductase; *Rv0098* and *Rv0099* both encode conserved hypothetical proteins of unknown function. FadD10 is one of a large number of fatty acid CoA-ligases proposed to be involved in lipid metabolism. The *nrp* gene encodes a non-ribosomal peptide synthase whose biological role is yet to be firmly elucidated, but it has been proposed to be involved in lipid metabolism due to its location in this operon (Cole *et al.*, 1998).

The *trxA* and *sodA* genes are both up-regulated in the mutant. The enzymes these genes encode play a role in



ORF	Fold induction	*P* value
Rv0096	12·4	<0·05
Rv0097	17·1	<0·05
Rv0098	15·9	<0·05
fadD10	6·3	0·07
Rv0100	5·2	<0·05
nrp	12·3	<0·05
Rv0102	1·5	0·3

Fig. 8. Operon structure of differentially expressed genes. The chromosomal arrangement of the *Rv0096* proposed operon is shown. Values below the genes show the mean fold increase in gene expression in the mutant strain and the *P* value from Student's *t*-test.

maintaining a suitable intracellular environment: thioredoxin participates in many redox reactions and maintains the redox potential of the cell, whilst superoxide dismutase is involved in the removal of free radicals generated during normal metabolism. The increase in these enzymes may indicate that the cell is under more stress than normal. Two genes associated with low oxygen environments are also induced, *cydB* encoding the cytochrome *d* ubiquinol oxidase subunit II and *ald* encoding L-alanine dehydrogenase. CydB has been proposed as the terminal oxidase complex which is used during low oxygen growth. Ald is well known to be induced in the Wayne model of hypoxic growth (Wayne & Sohaskey, 2001), under anaerobic conditions, and in stationary phase (Feng *et al.*, 2002) and may also be important in cell-wall biosynthesis where L-alanine is required for the peptidoglycan.

Of the other genes that show increased expression, citrate synthase 3 is involved in energy metabolism (tricarboxylic acid cycle) at an important control point of the cycle, *Rv0103c* encodes a probable copper cation transporter, three others are insertion sequence or phage elements (*Rv2424c*, *Rv2647*, *Rv3750c*), one is a bacteriocin-like protein (*Rv3660c*) and the remainder encode conserved hypotheticals or unknowns.

Taken together, these differences seem to suggest a subtle change in the normal metabolism (and growth) of the mutant bacteria and also a change in the type of intracellular stress that the bacteria are facing. A secondary effect of the mutation may be to reduce the growth rate, but it is not clear why.

Comparison of expression data

Several genes that were down-regulated appeared to be involved in normal growth; for example, several ribosomal proteins and *dnaB*. Several genes identified as stress proteins were also differentially expressed, either down-regulated (*grpE*, *ahpC* and *ahpD*) or up-regulated (*sodA*). This fits in with our previous observation that the mutant had a slight growth defect and indicated that the deletion of the system is probably causing some type of stress within the cells. It is possible that these genes are not directly controlled by RegX3 itself, but are down-regulated as a secondary effect of the mutation. To refine the list of potential regulon members, we conducted a meta-analysis of previously published data to determine if there were any significant patterns of expression relating to stress.

By comparing genes that were differentially expressed under various stress conditions, we looked for patterns of expression which would indicate that certain groups of genes are being co-ordinately regulated in response to any type of stress. We looked at their expression patterns in other published array data representing several different types of stress: heat shock (Stewart *et al.*, 2002), carbon limitation (Betts *et al.*, 2002), SDS treatment (Manganelli *et al.*, 2001), diamide treatment (Manganelli *et al.*, 2002), low oxygen tension (Sherman *et al.*, 2001) and low iron (Rodriguez *et al.*, 2002). Cluster analysis was used to group genes with similar patterns of expression. Genes which showed the same pattern of expression in more than one stress condition were in the same cluster and we considered them to be part of a general stress response. This made them unlikely to be directly controlled by RegX3, so we excluded them from our list. The remaining genes fell into four clusters.

Groups 1 and 2 were the up-regulated genes and groups 3 and 4 were the down-regulated genes. Two groups represented genes that only changed in the *senX3–regX3* mutant (1 and 3). The only condition in which *senX3* or *regX3* had been seen to change significantly was carbon starvation, where *senX3* was down-regulated by 2·2-fold, and one group (2) contained the genes that went up in both conditions. Group 4 contained those genes that were down-regulated in the mutant, but up-regulated under the other stress conditions (indicating that the change in expression was not due to stress alone). Of the 98 genes originally identified, we narrowed our list down to 50 (Table 3).

Thus, we predict that the genes in Table 3 are the most likely members of the *senX3–regX3* regulon. The majority of these genes are of unknown function, so at this stage it is difficult to speculate about the likely stimulus for this system.

Motif analysis

We further analysed the genes from Table 3 to see if there were any common motifs present in the regions immediately upstream of the genes that might be good candidates for a DNA-binding region for RegX3. We looked at a subset of those genes which showed the greatest-fold difference in expression. Sequences were analysed for the presence of tandem and inverted repeats and multiple alignments were carried out, but no significant patterns emerged. The sequences did not show any significant similarities with the previously identified binding site in the RegX3 promoter region (Himpens *et al.*, 2000). It may be that there are different DNA-binding recognition sites for RegX3 in alternative conformational states. Alternatively, it may be that these genes are controlled by an indirect effect and we would not expect to see RegX3 binding in that case.

Identification of the stimulus

To identify the stimulus to which the *senX3–regX3* system responds, we tried two approaches. First, we assayed the ability of the mutant to survive different *in vitro* conditions and stresses. We looked at viability during exposure to extremes of pH (2 and 12), ability to survive extended stationary phase in standing culture and ability to withstand complete nutrient starvation. The mutant showed no significant difference compared to the wild-type strain

Table 3. Cluster analysis of differentially regulated genes

Genes were grouped according to their behaviour under several different conditions. Group 1, up-regulated in Tame15 only; group 2, up-regulated in Tame15 and under carbon starvation; group 3, down-regulated in Tame15 only; group 4, down-regulated in Tame15, up-regulated in at least one other stress condition.

Group 1 ($n=16$)		Group 2 ($n=2$)		Group 3 ($n=34$)				Group 4 ($n=8$)	
ORF	Gene	ORF	Gene	ORF	Gene	ORF	Gene	ORF	Gene
Rv0096	*PPE1*	*Rv3750c*		*Rv0183*		*Rv1891*		*Rv0271c*	*fadE6*
Rv0097		*Rv3890c*		*Rv0312*		*Rv1942c*		*Rv0282*	
Rv0098				*Rv0430*		*Rv2040c*		*Rv0351*	*grpE*
Rv0101	*nrp*			*Rv0491*		*Rv2274c*		*Rv1346*	*fadE14*
Rv0103c	*ctpB*			*Rv0616c*		*Rv2308*		*Rv1592c*	
Rv0807				*Rv0647c*		*Rv2429*		*Rv1812c*	
Rv1089	*PE10*			*Rv0803*	*purL*	*Rv2430c*	*PPE41*	*Rv2428*	*ahpC*
Rv1622c	*cydB*			*Rv0867c*		*Rv2488c*		*Rv2760c*	
Rv2337c				*Rv0916c*	*PE7*	*Rv2594c*	*ruvC*		
Rv2424c	*IS*			*Rv0974c*	*accD2*	*Rv2650c*			
Rv2638				*Rv1148c*		*Rv2687c*			
Rv2647				*Rv1252c*	*lprE*	*Rv2757c*			
Rv2668				*Rv1330c*		*Rv2783c*	*gpsI*		
Rv2669				*Rv1355c*	*moeY*	*Rv2835c*	*ugpA*		
Rv3660c				*Rv1433*		*Rv2866*			
Rv3749c				*Rv1518*		*Rv3020c*	*PE28*		
				Rv1714		*Rv3100c*			

(data not shown). To survey a larger number of conditions, we then looked at promoter activity from pIKL-R1 under a variety of conditions to determine if it was induced. Conditions tested included several antibiotics (kanamycin, tetracycline, gentamicin, isoniazid, ampicillin, rifampicin), lysozyme, HCl, NaOH, DMSO, SDS and H_2O_2, but no conditions were found to up-regulate promoter activity. Thus, the stimulus for this system stills remains unknown.

Conclusion

We have constructed a strain with a deletion of the *senX3–regX3* 2CR. We used several different models to determine if the deletion of the *senX3–regX3* system had any effect on the virulence of the bacterium. The mutant showed significant attenuation in both activated and resting macrophages and in immunocompromised and immunocompetent mice. Thus, it seems that the mutant is less able to cause disease regardless of the involvement of the immune system. This attenuation was not as large as that seen previously for other mutants (e.g. the complete attenuation of the *trpD* mutant; Smith *et al.*, 2001), but it was a significant reduction. We used whole-genome microarrays to identify genes that are differentially expressed in the mutant, and based on these data we have identified 50 potential members of the *senX3–regX3* regulon. The construction of further mutants in these genes should lead to the identification of the genes whose roles are required during infection.

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