Molecular characterization of the mycobacterial SenX3–RegX3 two-component system: evidence for autoregulation

Sabine Himpens, Camille Locht and Philip Supply

Author for correspondence: Philip Supply. Tel: +33 3 20 87 11 54. Fax: +33 3 20 87 11 58.
e-mail: philip.supply@pasteur-lille.fr

INSERM U447, Institut Pasteur de Lille/Institut de Biologie de Lille, 1 rue du Professeur Calmette, F-59019 Lille Cedex, France

Environmental regulation of bacterial gene expression is often mediated by two-component signal transduction systems, which are themselves tightly regulated. The response regulator RegX3 and the cytoplasmic portion of the histidine kinase SenX3 from *Mycobacterium bovis* BCG were overproduced in *Escherichia coli* and purified as N-terminally (His)₆-tagged proteins. Phosphorylation assays demonstrated autophosphorylation of the cytoplasmic portion of SenX3 and a phosphotransfer from SenX3 to RegX3, involving conserved histidine and aspartate residues, respectively. In addition, as shown by electrophoretic mobility shift assays, (His)₆RegX3 was able to specifically bind to the promoter region of the *senX3-regX3* operon. Furthermore, operon fusion analyses indicated that the overexpression of the *senX3-regX3* operon increases the activity of the *senX3* promoter in *Mycobacterium smegmatis*. Together, these results indicate that the mycobacterial SenX3–RegX3 two-component system is positively autoregulated.

Keywords: two-component systems, autoregulation, phosphorylation, *Mycobacterium tuberculosis*

INTRODUCTION

Two-component systems provide a conserved mechanism for the coordinate regulation of gene expression in response to a wide variety of environmental input signals. Diverse processes such as chemotaxis, cell division, metabolic pathways, nitrogen fixation, stress responses and virulence are regulated by two-component systems (Stock *et al.*, 1989). Several hundred such systems have been identified in bacteria. They have been found in all prokaryotes examined to date, and some have been recently discovered in eukaryotes. In most systems, the response regulator is a transcriptional activator (for reviews see Alex & Simon, 1994; Hoch & Silhavy, 1995). The core mechanism of two-component systems consists of an autophosphorylation on a conserved histidine residue in the cytoplasmic domain of the sensor, followed by the transfer of the phosphate group onto a conserved aspartate residue of the response regulator. This results in the activation of the regulator leading to the appropriate response (Parkinson & Kofoid, 1992).

Autoregulation is an important feature of many two-component systems. It allows amplification of the response to help the cell to rapidly modify target gene expression when the appropriate signals are encountered. For instance, many two-component systems controlling virulence, quorum sensing, envelope stress response or sporulation control their own upregulation (e.g. Soncini *et al.*, 1995; Kleerebezem *et al.*, 1997; Rai-vio *et al.*, 1999). However, as shown for HilA, which regulates virulence factor genes in *Salmonella typhi-murium* (Bajaj *et al.*, 1996), autoregulation is not a universal rule.

The genome of *Mycobacterium tuberculosis* contains 30 genes encoding two-component-system proteins (Cole *et al.*, 1998). Their functions, regulation and the signals sensed by these systems are not known, and only three of them, MtrA–MtrB, SenX3–RegX3 and TrcS–TrcR, have been partially characterized (Supply *et al.*, 1997; Via *et al.*, 1996; Haydel *et al.*, 1999). The SenX3 sensor belongs to the PhoR and EnvZ subfamily and contains two hydrophobic, potential transmembrane regions in its N-terminal moiety, whereas the RegX3 response regulator belongs to the RO_{II} subfamily, of which PhoB and OmpR are the prototypes (Supply *et al.*, 1997).

Abbreviation: DIG, digoxigenin.

Here, we show that the cytoplasmic portion of SenX3 is able to autophosphorylate. The phosphate group is then transferred onto RegX3. This phosphorelay involves the conserved His-167 and Asp-52 residues of the transmitter domain of SenX3 and the receiver domain of RegX3, respectively. In addition, recombinant RegX3 is able to specifically bind the *senX3* promoter region, and overproduction of RegX3 increases the expression of the *senX3-regX3* operon in *Mycobacterium smegmatis*.

METHODS

Bacterial strains. All cloning steps were carried out in *Escherichia coli* XL-1 Blue (Stratagene). Recombinant SenX3 and RegX3 were expressed in *E. coli* M15 (Qiagen). Site-directed mutagenesis was performed in *E. coli* ES1301 *mutS* (Promega) followed by the amplification of the mutated plasmids in *E. coli* JM109 (Promega). The *M. smegmatis* mc²-155 strain (Jacobs *et al.*, 1991) was used for electroporation of the pREP vectors.

Reporter gene construction. The pREP7 vector containing the *senX3-lacZY* fusion has been described previously (Supply *et al.*, 1997). The pREP5 vector was constructed by inserting a 3.2 kb *HindIII-BamHI* fragment containing the *senX3-regX3* operon from pRegX3Bc1 (Supply *et al.*, 1997) into the unique *ScaI* site of pREP7.

β -Galactosidase assays. *M. smegmatis* was transformed with pREP5 or pREP7 as described by Kremer *et al.* (1995) and grown in Sauton medium supplemented with 25 μ g kanamycin ml⁻¹ until the OD₆₀₀ reached 0.2–0.4. The cells were then lysed by sonication and the β -galactosidase activity in the sonicate extracts was measured by the method of Miller (1992) as described previously (Supply *et al.*, 1997). β -Galactosidase units were calculated according to the formula $U = 1000 \times (OD_{420} - 1.75 \times OD_{550}) / [t \text{ (min)} \times \text{vol. (ml)} \times OD_{600}]$.

Production of recombinant proteins. To overproduce (His)₆RegX3, the *regX3* coding sequence was amplified by PCR from pRegX3Bc1 (Supply *et al.*, 1997) using synthetic oligonucleotides that included 5'-terminal *BamHI* half-sites (underlined). The sequences of these oligonucleotides were 5'-TCCATGACCAGTGTGTTGATTGTGCA-3' and 5'-TCCGCCCTCGAGTTTGTAGCCAC-3'. The PCR product was circularized by ligation, digested with *BamHI* to regenerate complete *BamHI* sites and cloned into the *BamHI* site of pQE30 (Qiagen). The stop codon, located 9 nt downstream of the *regX3* coding sequence, was provided by the vector sequence after digestion of the plasmid by *HindIII* and *SphI*, T4 DNA polymerase treatment and religation. Therefore, the recombinant (His)₆RegX3 contains an addition of three amino acids at its C-terminal end: Glu, Leu and Asn. To overproduce (His)₆SenX3, the *senX3* sequence encoding the cytoplasmic portion of SenX3 was amplified by PCR from pRegX3Bc1 using synthetic oligonucleotides that included 5'-terminal *BamHI* and *HindIII* restriction sites (underlined). The sequences of these oligonucleotides were 5'-GGGGATCCCCGGTTGCTGAGCGAGGAAGA-3' and 5'-CCCAAGCTTTCATCGGCTCAGCTCTTCCT-3'. The PCR product was digested with *BamHI/HindIII* and cloned into pQE30. The two products encoded by pQE30-RegX3 and pQE30-SenX3, named (His)₆RegX3 and (His)₆SenX3 respectively, contained a tag composed of 6 histidine residues at their N termini.

The mutant proteins (His)₆SenX3-H167Q and (His)₆RegX3-D52N were obtained using the pAlter site-directed muta-

genesis kit (Promega). The mutagenic oligonucleotides used had the following sequence: 5'-CGTCAGTCAAGAGCTC-AAG-3' and 5'-AGCATCAGATTGAGCAGGA-3', respectively. Codons 167 CAC and 52 GAT were modified to CAA and AAT, respectively. The mutated sequences were used to replace the wild-type sequences in the expression vectors pQE30-SenX3 and pQE30-RegX3. All the constructs were verified by sequencing using the ABI system (Perkin Elmer).

Purification of recombinant proteins. *E. coli* M15 cells containing the different expression vectors were grown in 500 ml LB medium containing 100 μ g ampicillin ml⁻¹ and 25 μ g kanamycin ml⁻¹. When the OD₆₀₀ reached 0.7–0.8, expression of the genes encoding the recombinant proteins was induced with 1 mM IPTG for 3 h 30 min. The cells were then harvested by centrifugation, resuspended in 5 ml buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0) per gram fresh weight and gently stirred for 1 h. After centrifugation at 10000 g for 15 min at 4 °C the supernatant was loaded onto a 4 ml Ni/NTA column (Qiagen). The column was first washed with buffer C (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 6.3) until the OD₂₈₀ became below 0.01, and then with 10 ml buffer D (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 5.9). The proteins were eluted with 10 ml buffer E (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 4.5). Two millilitre fractions were collected and analysed by SDS-PAGE and Coomassie blue staining. The purified proteins were dispensed in aliquots and stored at –20 °C. For small-scale preparations of the proteins, recombinant M15 cells containing the expression vectors were grown in 10 ml cultures and induced as described above. After centrifugation, the cells were resuspended in 2 ml buffer B. Three hundred microlitres of a 50% slurry of Ni/NTA resin was added to the supernatant after centrifugation at 10000 g for 15 min at 4 °C and the mixture was stirred for 30 min at room temperature. The resin was harvested by centrifugation at 15000 g for 10 s, washed three times with 1.5 ml buffer C, resuspended in 400 μ l buffer C containing 100 mM EDTA and gently mixed for 2 min at room temperature. This suspension was then centrifuged at 15000 g for 10 s. The supernatant was carefully removed and stored at –20 °C.

The purified proteins were renatured by two successive rounds of dialysis at 4 °C. Approximately 100 μ g proteins were first dialysed for 24 h against 150 ml PBS, 0.4 M sucrose, 0.4 M L-arginine, 1 mM EDTA (pH 8.0), and then against 200 ml PBS, 10% (v/v) glycerol (pH 8.0). The purified proteins were dispensed in aliquots and stored at –20 °C.

Phosphorylation assays. For phosphotransfer assays, 2 μ g renatured (His)₆SenX3 was incubated for 20 min at 25 °C with 10 μ Ci of [γ -³²P]ATP [6000 Ci mmol⁻¹ (222 TBq mmol⁻¹); Amersham] in a total volume of 20 μ l containing 10 mM MgCl₂, 25 mM Tris/HCl (pH 7.6). When indicated, 10 μ g purified (His)₆RegX3 was added to the reaction mixture and incubated for 15 min at 37 °C. The reaction was stopped by the addition of 10 μ l 3 $\times$ loading buffer (150 mM Tris/HCl, pH 6.8, 6% SDS, 0.3% bromophenol blue, 3% 2-mercaptoethanol, 30% v/v glycerol). The samples were loaded onto a 12% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred onto a nitrocellulose membrane. The membrane was dried and then exposed for autoradiography to an X-ray film.

For pulse-chase experiments, 10 μ g (His)₆SenX3 was incubated with 0.16 μ M [γ -³²P]ATP as described above, then 50 μ g purified (His)₆RegX3 and 320 μ M unlabelled ATP were simultaneously added to the reaction mixture. The standard

phosphorylation reaction was performed in 100 µl from which 20 µl aliquots were removed 0, 1, 5, 10 and 30 min after the addition of (His)₆RegX3 and unlabelled ATP. For each aliquot, the reaction was immediately stopped by the addition of 10 µl 3 × loading buffer. Alternatively, 10 µg purified (His)₆RegX3 and 0.16 µM [³²P]ATP were simultaneously added to the phosphorylation reaction mixture containing (His)₆SenX3 preincubated with 320 µM unlabelled ATP.

To test the sensitivity of the phosphorylated proteins to acidic and basic conditions, 2.5 µl 10% SDS and 2.5 µl 1 M HCl or 10 M NaOH were added to 20 µl of the phosphorylation reaction mixture and then incubated for 15 min at 37 °C. The samples were then dialysed against 1 M Tris/HCl (pH 6.8) before SDS-PAGE.

Electrophoretic mobility shift assays. DNA-binding activities of renatured (His)₆RegX3 were assessed by using digoxigenin (DIG)-labelled double-stranded DNA. Oligonucleotides 5'-GGGGTACCTTGTGTTGAGATCCCACCTGC-3' and 5'-GGGGTACCAAGGAAAATCCTACAAATCCGGTGA-3' were used to amplify a 189 bp DNA fragment upstream of the *senX3* gene. These oligonucleotides included terminal *KpnI* sites (underlined) to increase the efficiency of DIG labelling by the terminal transferase. The PCR product named 5'*senX3*-189 was digested with *KpnI* and labelled at its 3' ends with DIG-dideoxy-UTP using the DIG Oligonucleotide 3' End Labelling Kit (Boehringer Mannheim) as recommended by the supplier. The following DNA fragments were used as competitors in electrophoretic mobility shift assays: (1) an 85 bp fragment (5'*senX3*-85) corresponding to the 3' region of the 5'*senX3*-189 probe (obtained by PCR using the pair of oligonucleotides 5'-TGGCGTAGTGTGTGACTTGTC-3' and 5'-GGGGTACCAAGGAAAATCCTACAAATCCGGTGA-3'); (2) a 122 bp fragment (5'*senX3*-122) corresponding to the 5' region of the 5'*senX3*-189 probe (obtained by PCR using the pair of oligonucleotides 5'-GGGGTACCTTGTGTTGAGATCCCACCTGC-3' and 5'-AAGTCACACACTACGCCACAG-3'); and (3) a 42 bp double-stranded oligonucleotide (5'*senX3*-42) with the sequence 5'-ATGTGAACGGTAACCGAACAGCTGTGGCGTAGTGTGTGACTT-3' corresponding to an internal segment of the 5'*senX3*-189 probe.

Purified renatured (His)₆RegX3 was incubated for 20 min at room temperature with 5 ng DIG-labelled 5'*senX3*-189 (1.5 fmol) and 2 µl 5 × binding buffer (10 mM Tris/HCl, pH 8.0, 2 mM MgCl₂, 50 mM KCl, 1 mM DTT, 50%, v/v, glycerol, 0.05%, v/v, Nonidet P40) in a total volume of 10 µl. The samples were then loaded onto a 12% (w/v) polyacrylamide/45 mM Tris-borate/1 mM EDTA (pH 8.0) native gel. After electrophoresis, the DNA was blotted onto a positively charged nylon membrane (Boehringer Mannheim), fixed under UV light and developed using the Boehringer Mannheim Bioluminescence kit. The membrane was exposed for 20 min to 2 h for autoradiography to an X-ray film. When the effect of phosphorylation on binding was tested, (His)₆RegX3 was phosphorylated in the same phosphorylation buffer as above containing 500 µM unlabelled ATP.

RESULTS

Synthesis and purification of histidine-tagged RegX3 and SenX3

Full length RegX3 and the cytoplasmic portion of SenX3 were overproduced and purified as N-terminally (His)₆-tagged proteins. The cytoplasmic portion of SenX3 was

Fig. 1. Overproduction and purification of (His)₆RegX3 and (His)₆SenX3. (His)₆RegX3 and (His)₆SenX3 were overproduced in *E. coli* and purified on a Ni/NTA column as described in Methods. Crude lysates and purified fractions were subjected to SDS-PAGE and stained with Coomassie blue. (a) RegX3 purification. Lane 1, crude lysate of uninduced cells; lane 2, crude lysate of IPTG-induced cells; lane 3, 5 µg purified (His)₆RegX3. (b) SenX3 purification. Lane 1, 5 µg purified (His)₆SenX3; lane 2, crude lysate of uninduced cells; lane 3, crude lysate of IPTG-induced cells. The positions of (His)₆RegX3 and (His)₆SenX3 are indicated by the arrows. Molecular mass markers are indicated on the left.

chosen to avoid insolubility due to hydrophobic amino acid stretches and to thereby facilitate purification and biochemical analysis of this protein. After induction with IPTG, two major proteins of 31 kDa and 33 kDa were detected in crude lysates of the *E. coli* strains containing the RegX3 and the SenX3 expression vectors, respectively (Fig. 1). These proteins correspond to (His)₆RegX3 and (His)₆SenX3, as they were recognized by anti-His-tag antibodies (data not shown). The apparent molecular masses were somewhat higher than expected from the sequences (26.8 kDa and 32.1 kDa, respectively). This could at least partly be due to the presence of the polyhistidine tag, which may increase the apparent molecular mass by several kilodaltons.

The recombinant proteins were purified by using a nickel-chelate resin in the presence of 8 M urea. As shown in Fig. 1, purified (His)₆RegX3 and (His)₆SenX3 appeared as single bands on Coomassie blue-stained gels after electrophoresis in the presence of SDS. The recombinant proteins were then renatured by dialysis against a buffer containing 0.4 M sucrose and 0.4 M L-arginine to avoid proline *cis-trans* isomerization and to facilitate correct folding of the proteins (Bowden & Georgiou, 1990; Mukhopadhyay, 1997). L-Arginine and sucrose were subsequently eliminated by a second dialysis step.

Phosphotransfer between SenX3 and RegX3

The transduction of sensory signals by two-component systems generally occurs through a phosphorylation cascade. The (His)₆SenX3 protein was therefore tested

Fig. 2. Phosphorylation of (His)₆RegX3 and (His)₆SenX3. The purified proteins were incubated with [γ -³²P]ATP in 10 mM MgCl₂, 25 mM Tris/HCl (pH 7.6), as described in Methods. Lane 1, no protein; lane 2, 2 μ g (His)₆SenX3; lane 3, 10 μ g (His)₆RegX3; lanes 4–7, 2 μ g (His)₆SenX3 and 10 μ g (His)₆RegX3. After incubation, the proteins were treated with 1 M HCl (lane 5), 0.1 M HCl (lane 6) or 1 M NaOH (lane 7), as described in Methods.

for its capacity to be phosphorylated and to transfer the phosphate group to (His)₆RegX3. As shown in Fig. 2, (His)₆SenX3 was readily autophosphorylated in the presence of [γ -³²P]ATP. When (His)₆RegX3 was added to the SenX3 phosphorylation reaction mixture both proteins were phosphorylated. In the absence of (His)₆SenX3, no detectable (His)₆RegX3 phosphorylation occurred.

To determine whether RegX3 was phosphorylated directly from phosphorylated SenX3 or from the free nucleotide pool, (His)₆SenX3 was preincubated with 0.16 μ M [γ -³²P]ATP for 20 min, after which (His)₆RegX3 was added and incubation continued for various lengths of time. Phosphorylation of (His)₆RegX3 coincided with dephosphorylation of (His)₆SenX3 and the maximal level was reached within 5 min (Fig. 3c). Incorporation of ³²P into (His)₆RegX3 was not inhibited by the simultaneous addition of excess unlabelled ATP (Fig. 3b). In contrast, when (His)₆SenX3 was first incubated for 20 min with 320 μ M unlabelled ATP, followed by the addition of (His)₆RegX3 and 0.16 μ M [γ -³²P]ATP, efficient labelling of (His)₆SenX3 and (His)₆RegX3 was not detected (Fig. 3a). The competition by excess unlabelled ATP in the preincubation mixture indicates that the incorporation of label into (His)₆RegX3 depends on prior incorporation into (His)₆SenX3. Consistent with this result, the chase of label from (His)₆SenX3 to (His)₆RegX3 was not competed by excess unlabelled ATP, indicating that (His)₆RegX3 is phosphorylated by phosphotransfer from (His)₆SenX3 and not from the free nucleotide pool. These results also indicate that the cytoplasmic portion of SenX3 is sufficient for autophosphorylation and phosphotransfer to RegX3. Interestingly, both the (His)₆SenX3 and the (His)₆RegX3 phosphorylation were resistant to an

Fig. 3. Phosphotransfer from (His)₆SenX3 to (His)₆RegX3. (a) (His)₆SenX3 was preincubated for 20 min with 320 μ M unlabelled ATP. (His)₆RegX3 was then added together with 0.16 μ M [γ -³²P]ATP and incubation was continued for 0 (lane 1), 5 (lane 2), 10 (lane 3) or 30 min (lane 4). (b) (His)₆SenX3 was preincubated for 20 min with 0.16 μ M [γ -³²P]ATP. (His)₆RegX3 was then added together with 320 μ M unlabelled ATP, and incubation was continued for 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4) or 30 min (lane 5). (c) (His)₆SenX3 was preincubated for 20 min with 0.16 μ M [γ -³²P]ATP. (His)₆RegX3 was then added, and incubation was continued for 0 (lane 1), 1 (lane 2), 5 (lane 3), 10 (lane 4) or 30 min (lane 5).

excess of unlabelled ATP for at least up to 30 min (Fig. 3b), indicating that their phosphatidyl bond is rather stable.

Residues involved in the phosphotransfer reaction

Phosphorylation of acidic (aspartate, glutamate) residues can be distinguished from that of basic (arginine, histidine, lysine) residues by treatment with acid or base (Fujitaki & Smith, 1984). The phosphatidyl bonds of (His)₆SenX3 and (His)₆RegX3 were therefore tested for their chemical stability under acidic and basic conditions (Fig. 2, lanes 5–7). The phosphatidyl bond of (His)₆SenX3 was acid labile and base stable, suggesting that phosphorylation occurred at a basic residue, which in two-component sensor proteins is typically a histidine. The phosphatidyl bond of (His)₆RegX3 was base labile but stable under moderate acidic conditions, suggesting that phosphorylation occurred at an acidic residue, which in other regulators is typically an aspartate.

By analogy to other sensors and regulators, the conserved His-167 in (His)₆SenX3 and Asp-52 in (His)₆RegX3 (Swanson *et al.*, 1994; Supply *et al.*, 1997) were chosen to be altered by mutagenesis, and the recombinant mutant proteins (His)₆SenX3-H167Q and (His)₆RegX3-D52N were purified. When (His)₆SenX3-H167Q was incubated with [γ -³²P]ATP no autophosphorylation occurred (Fig. 4, lane 3), and the protein could not serve as phosphodonor for (His)₆RegX3 (Fig. 4, lane 7). (His)₆RegX3-D52N could not be phosphorylated, even in the presence of wild-type (His)₆SenX3 (Fig. 4, lane 8).

These results indicate that His-167 and Asp-52 are the sites of phosphorylation in SenX3 and RegX3, respect-

Fig. 4. Lack of phosphorylation of SenX3-H167Q and RegX3-D52N. (His)₆SenX3 or (His)₆SenX3-H167Q was incubated with [γ -³²P]ATP. (His)₆RegX3 or (His)₆RegX3-D52N was then added as described in Methods. Lane 1, no protein; lane 2, 2 µg (His)₆SenX3; lane 3, 2 µg (His)₆SenX3-H167Q; lane 4, 10 µg (His)₆RegX3; lane 5, 10 µg (His)₆RegX3-D52N; lane 6, 2 µg (His)₆SenX3 and 10 µg (His)₆RegX3; lane 7, 2 µg (His)₆SenX3-H167Q and 10 µg (His)₆RegX3; lane 8, 2 µg (His)₆SenX3 and 10 µg (His)₆RegX3-D52N.

ively, in agreement with the data obtained for other systems (Hess *et al.*, 1988; Igo & Silhavy, 1988; Ninfa & Bennett, 1991).

RegX3 specifically binds the *senX3* promoter region

Previous results have indicated that the *senX3*–*regX3* genes are expressed as a polycistronic operon, under the control of a promoter region upstream of the *senX3* gene (Supply *et al.*, 1997). The ability of (His)₆RegX3 to bind to this region was assessed by electrophoretic mobility shift assays using a 189 bp DIG-labelled PCR fragment containing nucleotides 52–225 and encompassing the *senX3* promoter region (Fig. 5a).

The data presented in Fig. 5 demonstrate specific DNA-binding activity of (His)₆RegX3 to this DNA fragment. Purified (His)₆RegX3 retarded the labelled probe (Fig. 5b). DNA binding was specific for the *senX3* promoter region, because the shifted band disappeared only in the presence of excess unlabelled DNA with the same sequence, but not in the presence of non-related DNA. Although the *senX3* and *regX3* genes are most likely polycistronic, the two genes are separated by a rather long intercistronic region composed of a class of duplicated sequences named mycobacterial interspersed repetitive units (MIRUs; Supply *et al.*, 1997), which could be conceivably involved in the regulation of the downstream gene. Therefore, electrophoretic mobility shift assays were carried out to test whether this intercistronic region could be bound by RegX3. This region was not retarded by (His)₆RegX3 (not shown), which suggests that it does not contain regulatory sequences recognized by RegX3.

Several additional competition assays with sequences internal to the 189 bp PCR fragment were carried out in order to define more precisely the (His)₆RegX3-binding site. A 122 bp fragment containing the 5' part of this region (from position 52 to 166) displaced the regulator from its labelled target, whereas an 85 bp fragment composed of the 3' part of this region (from 148 to 225) did not (Fig. 5a). Region 107–123 between the upstream *pgm* gene and *senX3* corresponds to a short palindromic sequence which might function as a transcriptional terminator of the *pgm* gene. Therefore, we reasoned that the (His)₆RegX3-binding site might lie 3' of this potential stem-loop structure. A 42 bp double-stranded oligonucleotide corresponding to segment 124–165 was thus tested and found to compete for the 189 bp labelled

Fig. 5. Binding activity of RegX3 to the *senX3* promoter region. (a) The sequence of the *senX3* promoter region. The 189 bp DIG-labelled DNA fragment corresponds to region 52–225. The 85 bp fragment is shown by a dotted line, the 122 bp fragment by a dashed line and the 42 bp fragment by a continuous line. The putative Shine–Dalgarno (SD) and –10 box sequences are underlined. The inverted arrows indicate a palindromic sequence. (b) Electrophoretic mobility shift assays. One and a half femtomoles of 5' *senX3*-189 was mixed with purified (His)₆RegX3 in 10 µl binding buffer as described in Methods. The binding mixture contained 15 pmol (His)₆RegX3 alone, or with a 50× excess of specific unlabelled 5' *senX3*-189 (S) or a 100× excess of poly(dIdC) nonspecific DNA (NS) (lanes 2, 3 and 4, respectively). Lane 1 contained no protein. The positions of the well (W), the complexed DNA (C) and the free DNA (F) are indicated on the left.

target sequence, indicating that this 42 bp fragment contains the RegX3 binding site (Fig. 5a). In addition to indicating specificity of the (His)₆RegX3-binding activity these results also suggest that the *senX3-regX3* operon is autoregulated.

Increase of the *senX3* promoter activity by overexpression of *senX3-regX3* in *M. smegmatis*

In vitro binding of RegX3 to the *senX3-regX3* promoter region suggests that *senX3-regX3* is autoregulated. To assess whether this system is positively or negatively autoregulated, the complete operon was cloned into a mycobacterial shuttle vector together with the *senX3* promoter region fused to the promoterless *lacZ* reporter gene (yielding pREP5), and introduced into *M. smegmatis*. After growth in liquid medium, β -galactosidase activities measured in extracts of cells containing pREP5 were reproducibly more than two-fold higher than those obtained in extracts of cells containing pREP7, a plasmid that bears the *senX3* promoter region fused with *lacZ* in the absence of the *senX3-regX3* operon (the mean values $\pm$ standard deviation of β -galactosidase activities of four independent assays were 39 ± 5 units, compared to 17 ± 4 units, respectively). These results indicate that the *senX3-regX3* operon is positively regulated by its own regulator.

DISCUSSION

The general mechanism of communication in two-component systems involves successive phosphorylation of the two partners. Sensors autophosphorylate on a conserved histidine residue using ATP as the phosphodonator. The phosphate group is then transferred onto a conserved aspartate residue of the response regulator (Parkinson & Kofoed, 1992; Stock *et al.*, 1995). Our results indicate that the same mechanism prevails for the mycobacterial SenX3-RegX3 system. A truncated form of the SenX3 sensor containing the cytoplasmic C-terminal portion of the protein was found to be sufficient to carry out the autophosphorylation reaction, as has been observed for other sensors (Roberts *et al.*, 1994). The cytoplasmic domain of SenX3 contains His-167 found to be critical for phosphorylation, as well as the typical motifs of the kinase domain (Parkinson & Kofoed, 1992).

Sequence similarity analysis ties RegX3 to the RO_{II} subfamily of response regulators (Parkinson & Kofoed, 1992), which are presumed or demonstrated transcriptional regulators. The C terminus of RegX3 contains a putative helix-turn-helix DNA-binding motif, similar to that recently identified in the OmpR structure (Kondo *et al.*, 1997; Martinez-Hackert & Stock, 1997). Electrophoretic mobility shift assays showed that RegX3 specifically binds the *senX3* promoter region. Furthermore, operon fusion analyses indicated that activity of the *senX3* promoter is significantly increased by the expression of the *senX3-regX3* operon itself. Taken

together, these two results indicate that the SenX3-RegX3 system is positively autoregulated and suggest that the response mediated by this system is subject to autoamplification.

In addition to their autokinase activity using their partner sensors as phosphodonors, some response regulators also possess an autophosphatase activity, resulting in half-lives of their phosphate group of a few seconds or minutes (Hess *et al.*, 1988; Makino *et al.*, 1989). In other systems, it is the cytoplasmic transmitter domain of the sensor that exerts phosphatase activity on the cognate response regulator. In that case, the dephosphorylation of the regulator is stimulated by the presence of ATP (Aiba *et al.*, 1989; Igo *et al.*, 1989). Autoactivation combined with the phosphatase activities allows for a temporary amplification of the response. Such a mechanism may be important for systems controlling responses to transient stresses and properly timed expression of the target genes (Raivio *et al.*, 1999). As far as SenX3-RegX3 is concerned, we found that after rapid initial phosphotransfer from phosphorylated SenX3 to RegX3, the phosphate group remained stably attached to RegX3 for at least 30 min, even in the presence of excess ATP. From these observations we infer that neither RegX3 or SenX3 contain a strong phosphatase activity, at least under the experimental conditions used here. This suggests that the modulation of the adaptive response of the SenX3-RegX3 system may be rather slow, which is perhaps related to the general slow growth rate of mycobacteria. Alternatively, RegX3 dephosphorylation may be catalysed by auxiliary phosphatases as has been shown for the *Bacillus subtilis* Spo system (Perego *et al.*, 1994).

For some response regulators, phosphorylation appears to be an absolute requirement for detectable binding to specific DNA targets (Boucher & Stibitz, 1995; Li *et al.*, 1994). In many other cases, phosphorylation increases the affinity for the target DNA by a factor of 10–100, although the unphosphorylated response regulator retains some DNA-binding activity (Aiba *et al.*, 1989; Makino *et al.*, 1989; Forst *et al.*, 1989; Nakashima *et al.*, 1991; Boucher *et al.*, 1994; Hoch & Silhavy, 1995; Lynch & Lin, 1996; Dahl *et al.*, 1997; Meyer *et al.*, 1997). Our results indicate that phosphorylation is not essential for RegX3 binding. The observed binding of the non-phosphorylatable mutant (His)₆RegX3-D52N rules out effect of phosphorylation of recombinant RegX3 from non-cognate sensors in *E. coli*. Moreover, we observed no detectable effect of (His)₆RegX3 phosphorylation on binding to the *senX3-regX3* operon compared to unphosphorylated (His)₆RegX3 (not shown). These results suggest that phosphorylation of RegX3 may exert its effect predominantly on protein-protein interactions stimulating transcription, rather than on DNA binding. For several two-component systems it is known that, in addition to its effect on DNA binding, phosphorylation may act by favouring protein-protein interactions within protein-DNA transcription complexes (Weiss *et al.*, 1992; Porter *et al.*, 1993; Bird *et al.*, 1996; Wyman *et al.*, 1997; Boucher *et al.*, 1997).

Interestingly, a predominant role of phosphorylation on protein–protein interaction has also been proposed for PhoP of *B. subtilis*, which is a close homologue of RegX3. This protein also binds its DNA targets regardless of its phosphorylation state, and phosphorylation only marginally affects DNA-binding affinity (Liu & Hulett, 1997). Alternatively, the above observations may reflect differences in affinities between the promoter of the two-component system operon and the promoters of other members of the regulon for the phosphorylated regulator, as shown for the *Bordetella pertussis* BvgA regulator (Steffen *et al.*, 1996). In that case, different effects of phosphorylation on binding to various target promoters may offer a wider range of regulation levels and potentially allow for differential expression of the regulon members during the pathway activation (Raivio *et al.*, 1999).

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REFERENCES

- Aiba, H., Nakasai, F., Mizushima, S. & Mizuno, T. (1989). Phosphorylation of a bacterial activator protein, OmpR, by a protein kinase, EnvZ, results in stimulation of its DNA-binding ability. *J Biochem* **109**, 5–7.
- Alex, L. A. & Simon, M. I. (1994). Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. *Trends Genet* **10**, 133–138.
- Bajaj, V., Lucas, R. L., Hwang, C. & Lee, C. A. (1996). Co-ordinate regulation of the *Salmonella typhimurium* invasion genes by environmental and regulatory factors is mediated by control of *hila* expression. *Mol Microbiol* **22**, 703–714.
- Bird, T. H., Grimsley, J. K., Hoch, J. A. & Spiegelman, G. B. (1996). The *Bacillus subtilis* response regulator SpoOA stimulates transcription of the *spoII*G operon through modification of RNA polymerase promoter complexes. *J Mol Biol* **256**, 436–448.
- Boucher, P. E. & Stibitz, S. (1995). Synergistic binding of RNA polymerase and BvgA phosphate to the pertussis toxin promoter of *Bordetella pertussis*. *J Bacteriol* **177**, 6486–6491.
- Boucher, P. E., Menozzi, F. D. & Locht, C. (1994). The modular architecture of bacterial response regulators: insights into the activation mechanism of BvgA transactivator of *Bordetella pertussis*. *J Mol Biol* **241**, 363–377.
- Boucher, P. E., Murakami, K., Ishihama, A. & Stibitz, S. (1997). Nature of DNA binding and RNA polymerase interaction of the *Bordetella pertussis* BvgA transcriptional activator at the *fha* promoter. *J Bacteriol* **179**, 1755–1763.
- Bowden, G. A. & Georgiou, G. (1990). Folding and aggregation of β -lactamase in the periplasmic space of *Escherichia coli*. *J Biol Chem* **265**, 16760–16766.
- Cole, S., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Dahl, J. L., Wei, B. & Kadner, R. J. (1997). Protein phosphorylation affects binding of the *Escherichia coli* transcription activator UhpA to the *uhpT* promoter. *J Biol Chem* **272**, 1910–1919.
- Forst, S., Delgado, J. & Inouye, M. (1989). Phosphorylation of OmpR by the osmosensor EnvZ modulates expression of the *ompF* and *ompC* genes in *Escherichia coli*. *Proc Natl Acad Sci USA* **86**, 6052–6056.
- Fujitaki, J. M. & Smith, R. A. (1984). Techniques in the detection and characterization of phosphoramidate-containing proteins. *Methods Enzymol* **107**, 23–36.
- Haydel, S. E., Dunlap, N. E. & Benjamin, W. H., Jr (1999). *In vitro* evidence of two-component system phosphorylation between the *Mycobacterium tuberculosis* TcR/TcS proteins. *Microb Pathog* **26**, 195–206.
- Hess, J. F., Bourret, R. B. & Simon, M. I. (1988). Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature* **336**, 139–143.
- Hoch, J. A. & Silhavy, T. J. (1995). *Two-Component Signal Transduction*. Washington, DC: American Society for Microbiology.
- Igo, M. M. & Silhavy, T. J. (1988). EnvZ, a transmembrane environmental sensor of *Escherichia coli* K-12, is phosphorylated *in vitro*. *J Bacteriol* **170**, 5971–5973.
- Igo, M. M., Ninfa, A. J., Stock, J. B. & Silhavy, T. J. (1989). Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. *Genes Dev* **3**, 1725–1734.
- Jacobs, W. B., Jr, Kalpana, G. V., Cirillio, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G. & Bloom, B. R. (1991). Genetic systems for mycobacteria. *Methods Enzymol* **204**, 537–555.
- Kleerebezem, M., Quadri, L. E., Kuipers, O. P. & De Vos, W. M. (1997). Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol Microbiol* **25**, 895–904.
- Kondo, H., Nakagawa, A., Nishihira, J., Nishimura, Y., Mizuno, T. & Tanaka, I. (1997). *Escherichia coli* positive regulator OmpR has a large loop structure at the putative RNA polymerase interaction site. *Nat Struct Biol* **4**, 28–31.
- Kremer, L., Baulard, A., Estaquier, J., Poulain-Godefroy, O. & Locht, C. (1995). Green fluorescent protein as a new expression marker in mycobacteria. *Mol Microbiol* **17**, 913–922.
- Li, J., Kustu, S. & Stewart, V. (1994). *In vitro* interaction of nitrate-responsive regulatory protein NarL with DNA target sequences in the *fdnG*, *narG*, *narK* and *frdA* operon control regions of *Escherichia coli* K-12. *J Mol Biol* **241**, 150–165.
- Liu, W. & Hulett, F. M. (1997). *Bacillus subtilis* PhoP binds to the *phoB* tandem promoter exclusively within the phosphate starvation-inducible promoter. *J Bacteriol* **179**, 6302–6310.
- Lynch, A. S. & Lin, E. C. C. (1996). Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. *J Bacteriol* **178**, 6238–6249.
- Makino, K., Shinagawa, H., Amemura, M., Kawamoto, T., Yamada, M. & Nakata, A. (1989). Signal transduction in the phosphate regulon of *Escherichia coli* involves phosphotransfer between PhoR and PhoB proteins. *J Mol Biol* **210**, 551–559.
- Martinez-Hackert, E. & Stock, A. M. (1997). Structural relationships in the OmpR family of winged-helix transcription factors. *J Mol Biol* **269**, 301–312.
- Meyer, M., Dimroth, P. & Bott, M. (1997). *In vitro* binding of the response regulator CitB and of its carboxy-terminal domain to A+T-rich DNA target sequences in the control region of the

- divergent *citC* and *citS* operons of *Klebsiella pneumoniae*. *J Mol Biol* **269**, 719–731.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: a Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mukhopadhyay, A. (1997). Inclusion bodies and purification of proteins in biologically active forms. *Adv Biochem Eng Biotechnol* **56**, 61–109.
- Nakashima, K., Kanamaru, K., Aiba, H. & Mizuno, T. (1991). Signal transduction and osmoregulation in *Escherichia coli*. *J Biol Chem* **266**, 10775–10780.
- Ninfa, A. J. & Bennet, R. L. (1991). Identification of the site of autophosphorylation of the bacterial protein kinase/phosphatase NR11. *J Biol Chem* **266**, 6888–6893.
- Parkinson, J. S. & Kofoid, E. C. (1992). Communication modules in bacterial signalling proteins. *Annu Rev Genet* **26**, 71–112.
- Perego, M., Hanstein, C., Welsh, K. M., Djavakhishvili, T., Glaser, P. & Hoch, J. A. (1994). Multiple protein-aspartate phosphatases provide a mechanism for the integration of diverse signals in the control of development in *B. subtilis*. *Cell* **79**, 1047–1055.
- Porter, S. C., North, A. K., Wedel, A. B. & Kustu, S. (1993). Oligomerization of NtrC at the *glnA* enhancer is required for transcriptional activation. *Genes Dev* **7**, 2258–2273.
- Raivio, T. L., Popkin, D. L. & Silhavy, T. J. (1999). The Cpx envelope stress response is controlled by amplification and feedback inhibition. *J Bacteriol* **181**, 5263–5272.
- Roberts, D. L., Bennett, D. W. & Forst, S. A. (1994). Identification of the site of phosphorylation on the osmosensor, EnvZ, of *Escherichia coli*. *J Mol Biol* **269**, 8728–8733.
- Soncini, F. C., Vescovi, E. G. & Groisman, E. A. (1995). Transcriptional autoregulation of the *Salmonella typhimurium phoPQ* operon. *J Bacteriol* **177**, 4364–4371.
- Steffen, S., Goyard, P. & Ullmann, A. (1996). Phosphorylated BvgA is sufficient for transcriptional activation of virulence-regulated genes in *Bordetella pertussis*. *EMBO J* **15**, 102–109.
- Stock, J. B., Ninfa, A. J. & Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev* **53**, 450–490.
- Stock, J. B., Surette, M. G., Levit, M. & Park, P. (1995). Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In *Two-Component Signal Transduction*, pp. 25–52. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.
- Supply, P., Magdalena, J., Himpens, S. & Locht, C. (1997). Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. *Mol Microbiol* **26**, 991–1003.
- Swanson, R. V., Alex, L. A. & Simon, M. I. (1994). Histidine and aspartate phosphorylation: two-component systems and the limits of homology. *Trends Biochem Sci* **19**, 485–490.
- Via, L. E., Curcic, R., Mudd, M. H., Dhandayuthapani, S., Ulmer, R. J. & Deretic, V. (1996). Elements of signal transduction in *Mycobacterium tuberculosis*: *in vitro* phosphorylation and *in vivo* expression of the response regulator MtrA. *J Bacteriol* **178**, 3314–3321.
- Weiss, V., Claverie-Martin, F. & Magasanik, B. (1992). Phosphorylation of nitrogen regulator I of *Escherichia coli* induces strong cooperative binding to DNA essential for activation of transcription. *Proc Natl Acad Sci U S A* **89**, 5088–5092.
- Wyman, C., Rombel, I., North, A. K., Bustamante, C. & Kustu, S. (1997). Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science* **275**, 1658–1661.

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