The AraC Family Transcriptional Regulator Rv1931c Plays a Role in the Virulence of Mycobacterium tuberculosis†
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A Mycobacterium tuberculosis strain disrupted in the AraC homologue Rv1931c was isolated. The mutant strain exhibited reduced survival both in macrophages and in a mouse infection model, with survival being restored on complementation with the Rv1931c gene. These results suggest that Rv1931c regulates genes important for virulence of M. tuberculosis.

Transcriptional regulators of the AraC family are widespread among bacteria and regulate genes having diverse functions, ranging from carbon metabolism to stress responses to virulence (4, 7). Six members of this family (Rv1317c [alkA], Rv1395, Rv1931c, Rv3082c [virS], Rv3736, and Rv3833) have been identified in the genome of Mycobacterium tuberculosis by sequence similarity to the defining DNA binding domain. The first of these, Rv1317c (alkA), has additional domains conferring a role in the repair of alkylated DNA, with the regulatory functions residing at the N terminus. A mutant strain of H37Rv containing a defined mutation in Rv1317c, which also affects the downstream gene Rv1316c, did not show any significant differences in bacillary loads in the lungs, liver, or spleen following infection of BALB/c mice (3). The remaining five homologues possess the AraC family motif at their C termini, a typical location for this family of regulatory proteins, and appear to be straightforward transcriptional regulators. Interestingly, a transposon mutation was identified in one of these, Rv1395, from a signature-tagged mutagenesis screen of strain Mt103, which was confirmed to show attenuation in lungs upon infection of BALB/c mice with the individual strain (1). Subsequently, Rv1395 has been shown to regulate the expression of a cytochrome P450-encoding gene transcribed divergently from it (10). Another member of this AraC family, Rv3082c (virS), has been suggested to play a role in M. tuberculosis pathogenesis on the basis of sequence similarities (5) and has also been shown to regulate an operon transcribed divergently from it (15). The AraC homologue Rv1931c is annotated as a probable transcriptional regulator in the TubercuList database (http://genolist.pasteur.fr/TubercuList) and has been predicted to be nonessential by Himar 1-based transposon mutagenesis (6). A DNA fragment containing the coding sequence of the target gene along with approximately 2 kb of the flanking region was generated by PCR from M. tuberculosis genomic DNA and cloned into Escherichia coli plasmid pUC19. Four hundred ninety-four base pairs of the 777-bp coding sequence of Rv1931c was then deleted, and a unique cloning site was simultaneously introduced by inverse PCR and religation of the PCR product. A kanamycin resistance gene from pUC4K was then inserted into this site. Finally, the disrupted fragment containing the flanking regions plus the kan gene was cloned into the streptomycin counterselection vector ptrpA-1-rpsL∗ (12).

The targeting construct was electroporated into M. tuberculosis strain 1424, a streptomycin-resistant derivative of H37Rv (2). Both one-step and two-step selections were performed by streptomycin selection as previously described (12, 13). Colonies obtained conforming to the desired phenotype were further screened by PCR (data not shown) and Southern analyses (Fig. 1) to identify genuine double-crossover recombinants. In addition, the genotype of the isolate selected for further study was further confirmed with microarrays (data not shown).

In a preliminary experiment to assess the effects of the mutation obtained on M. tuberculosis pathogenesis, the mutant strain was compared with wild-type strain H37Rv in a mouse model of infection. Groups of BALB/c mice were infected intravenously with approximately 5 × 10⁸ cells of each strain, and the progress of the infection was assessed by determining CFU counts at various time points. Mice infected with the wild-type strain developed clinical symptoms of disease between days 69 and 133 postinfection and had to be sacrificed. In contrast, all mice infected with ΔRv1931c were still surviving at day 133 postinfection, and CFU counts from both lungs and spleens of mice infected with this mutant were approximately 1 logarithm lower than those obtained following infection with the wild type, with counts from spleens decreasing after 69 days (data not shown), although growth under normal culture conditions in vitro was indistinguishable from that of the wild type.

To confirm that the attenuation seen in the initial experiment with ΔRv1931c was due to the introduced mutation, it was necessary to both complement the mutation with the cloned Rv1931c gene and compare the progress of infection...
caused by these strains with that of the isogenic wild-type strain, 1424. Therefore, a 968-bp fragment of DNA containing the entire \( \text{Rv1931c} \) coding sequence plus 160 bp of the upstream sequence was cloned into mycobacterial integrating vector pKP201. This plasmid carries a hygromycin resistance gene and the \( \text{attP} \) site but lacks the integrase, which must be supplied in trans for integration to occur; this function is provided by cotransformation with pBluescript \( \text{int} \) (16), a plasmid that fails to replicate in mycobacteria, resulting in increased stability of the clone. The insert in the complementing clone, pCF51, was sequenced to confirm that no mutations had been introduced. Following introduction of pCF51 into the \( \Delta \text{Rv1931c} \) strain, RNA was isolated and comparative expression analysis with microarrays revealed that the inserted gene was expressed (data not shown). A second mouse infection experiment was conducted as before but with strains 1424 (wild type), \( \Delta \text{Rv1931c} \), and \( \Delta \text{Rv1931c::pCF51} \). The growth of wild-type strain 1424 was similar to that seen previously with H37Rv (11), although a direct comparison was not made. Although it appeared that the mutant strain grew better than the wild type initially, we believe this to be an artifact as the difference between the mutant and the wild type at day 21 is not significant by \( t \) test \((P > 0.05)\) and in the preliminary experiment described above this was not observed. Overall a level of attenuation similar to that seen in the previous experiment was observed with the \( \Delta \text{Rv1931c} \) mutant, whereas with the complemented strain the bacterial loads in the lungs and spleen resembled those obtained with the wild-type strain (Fig. 2), confirming that the phenotype seen was due to the disruption of the \( \text{Rv1931c} \) gene.

To further assess the phenotype of the \( \Delta \text{Rv1931c} \) mutant strain, strains 1424 (wild-type), \( \Delta \text{Rv1931c} \), and \( \Delta \text{Rv1931c::pCF51} \) were used to infect mouse bone marrow-derived macrophages in vitro and the numbers of CFU were determined at

**FIG. 1.** Identification of an \( \text{Rv1931c} \) mutant by Southern hybridization. At the top a schematic representation of the gene locus is shown in which the upper part shows the arrangement in the mutant and the lower part shows that in the wild type. The cloned region is shown as a thick black line, and the introduced kanamycin resistance gene in the mutant is shown as a white box within this. The locations of the hybridizing probes (small solid bars) and the expected sizes of the hybridizing fragments for each of the 5' and 3' Southern blots are indicated. Genomic DNAs isolated from the mutant strain (KO) and the parental wild-type (WT) strain were digested with ClaI, transferred to nylon membrane, and hybridized to radioactively labeled DNA probes. The sizes of the hybridizing bands were determined from the migration distances of the DNA molecular size markers \( \lambda \)-HindIII+EcoRI and \( \lambda \)-HindIII.

**FIG. 2.** Phenotype of the \( \Delta \text{Rv1931c} \) mutant and complemented strains in a mouse model of infection. BALB/c mice were inoculated intravenously with approximately \( 5 \times 10^5 \) CFU of each strain. The survival and multiplication of the \( M. \text{tuberculosis} \) strains in the lungs and the spleen were determined by CFU counts and are shown for the 1424 wild-type (○), \( \Delta \text{Rv1931c} \) (▼), and \( \Delta \text{Rv1931c::pCF51} \) (■) strains. The results for each time point are the means of CFU determinations performed on organs from three mice, and the error bars show the standard deviations. The asterisk indicates that the result is statistically significantly different from that of the wild type by the two-tailed Student \( t \) test for groups of unequal variance \((P < 0.01)\), as well as by single-factor analysis of variance \((P < 0.01)\). KO, knockout.
various time points following infection. Bone marrow cells were extracted from the hind leg bones of 6- to 8-week-old female BALB/c mice and cultured in Iscove’s modified Dulbecco’s medium supplemented with 5% heat-inactivated fetal calf serum, 2 mM glutamine, 80 μM 2-mercaptoethanol, and 10% supernatant of L929 cells to provide macrophage colony-stimulating factor in 12-well plates for 5 days. Following this, cells were infected for 6 h at a multiplicity of infection of one bacterium to two macrophages with each strain. The medium was removed, the cells were washed with prewarmed medium, and fresh medium was added. At selected time points samples were lysed with 2% saponin and viable bacterial counts were determined by plating serial dilutions on Middlebrook 7H11 agar plates. Again the ΔRv1931c mutant exhibited a reduced ability to survive and replicate in comparison with the wild type, yielding CFU counts approximately 1 logarithm lower than the Rv1931c-complemented strain in each replicate experiment with similar results; the results of a representative experiment are shown. The results for each time point are the means of CFU determinations performed on triplicate infections, and the error bars show the standard deviations. The asterisk indicates that the result is statistically significantly different from that of the wild type by the two-tailed Student t test for groups of unequal variance (*P < 0.01), as well as by single-factor analysis of variance (P < 0.01).

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