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FEMS Microbiology Letters 231 (2004) 237–245

FEMS MICROBIOLOGY Letters

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Disruption of response regulator gene, *devR*, leads to attenuation in virulence of *Mycobacterium tuberculosis*

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Received 26 February 2003; received in revised form 16 October 2003; accepted 17 December 2003

First published online 17 January 2004

Abstract

The *devR-devS* two-component system of *Mycobacterium tuberculosis* was identified earlier and partially characterized in our laboratory. A *devR::kan* mutant of *M. tuberculosis* was constructed by allelic exchange. The *devR* mutant strain showed reduced cell-to-cell adherence in comparison to the parental strain in laboratory culture media. This phenotype was reversed on complementation with a wild-type copy of *devR*. The *devR* mutant and parental strains grew at equivalent rates within human monocytes either in the absence or in the presence of lymphocytic cells. The expression of DevR was not modulated upon entry of *M. tuberculosis* into human monocytes. However, guinea pigs infected with the mutant strain showed a significant decrease in gross lesions in lung, liver and spleen; only mild pathological changes in liver and lung; and a nearly 3 log lower bacterial burden in spleen compared to guinea pigs infected with the parental strain. Our results suggest that DevR is required for virulence in guinea pigs but is not essential for entry, survival and multiplication of *M. tuberculosis* within human monocytes in vitro. The attenuation in virulence of the *devR* mutant in guinea pigs together with DevR-DevS being a bona fide signal transduction system indicates that DevR plays a critical and regulatory role in the adaptation and survival of *M. tuberculosis* within tissues.
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Keywords: DevR-DevS two-component system; *Mycobacterium tuberculosis devR* mutant; Attenuation of virulence

1. Introduction

The *Mycobacterium tuberculosis* genome encodes 11 complete two-component systems and seven orphan signal transduction proteins [1]. While four of them, MtrA [2], SenX3-RegX3 [3], TrcS-TrcR [4] and DevR-DevS [5], have been established as authentic two-component systems based on their biochemical properties, their role in regulating various cellular functions is just beginning to be elucidated. MtrA is essential [6] and other response regulators, PhoP, PrrA and MprA, have been implicated in virulence [7], adaptation of tubercle bacilli in an early phase of intracellular growth [8], and the establishment and maintenance of persistent infection [9], respectively. Likewise, our understanding of the environmental cues sensed by these two-component systems is quite preliminary. Bacterial entry into macrophages was reported to lead to alterations in the activity of several response regulator genes [9]. Hypoxia was recognized as a key signal that led to extensive, rapid and dramatic changes in gene expression including the induction of the *devR-devS* two-component system [10].

The DevR-DevS (Rv3133c-Rv3132c) system was first identified in our laboratory [11] by a subtractive hybridization strategy employed with the specific intention of identifying virulence genes of *M. tuberculosis* [12]. The *devR-devS* genes encode a response regulator, DevR, and

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doi:10.1016/S0378-1097(04)00002-3

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a histidine sensor kinase, DevS, respectively, that display phosphorylation properties typical of two-component system proteins [5]. Here we demonstrate the participation of DevR in the virulence of *M. tuberculosis*. Briefly, a *devR::kan* mutant strain of *M. tuberculosis* was constructed and characterized with respect to its morphology in laboratory media and within human monocytes (MN) and its contribution to the pathogenesis of *M. tuberculosis* infection using guinea pigs. The mutant was complemented with a wild-type copy of the *devR* gene resulting in the reversion of dispersed culture phenotype in laboratory culture media.

2. Materials and methods

2.1. Construction of devR mutant and complemented strains of M. tuberculosis

The *devR* gene, located in a ~3.3-kb *Eco*RI-*Hin*dIII insert of plasmid pJT53.34 [11], was disrupted with a blunt-ended kanamycin resistance cassette (*kan*) from pGP1-2 (kind gift from Dr. S. Tabor, Harvard Medical School, Boston, MA, USA) at a unique *Ppu*MI site. The disrupted *devR* allele was cloned into the corresponding site of plasmid pJQ200SK [13] to yield pJQ200SK*devR::kan* that was electroporated into *M. tuberculosis* H37Rv (ATCC #25618, Rockville, MD, USA) as described [14]. Single crossover transformants indicative of plasmid integration were selected on Middlebrook (MB) 7H10 agar containing kanamycin (20 μg ml^{-1}) and analyzed by polymerase chain reaction (PCR) for the presence of *devR*, *kan* and *sacB* gene sequences. An *M. tuberculosis* strain having a tandem duplication at the *devR* locus (called Dup *devR*) was subjected to sucrose selection for detection of the second crossover event. Briefly, Dup *devR* was propagated in MB 7H9 containing kanamycin (20 μg ml^{-1}) and subsequently in MB 7H9 containing kanamycin (20 μg ml^{-1}) and 2% sucrose and plated on MB 7H10 agar containing kanamycin (20 μg ml^{-1}) and 2% sucrose. Km^R and Suc^R transformants were further screened by PCR for the presence of *devS*, *kan* and *sacB* sequences. Disruption of *devR* by allelic exchange was confirmed by Southern hybridization. The mutant strain was complemented by electroporation of plasmid pDSdevR containing a ~1-kb fragment comprising the complete *devR* gene and 327-bp upstream promoter region in pFPV-Hyg. pFPV-Hyg was modified from pFPV27 (generous gift from Dr. L. Ramakrishnan, University of Washington, Seattle, WA, USA) by introduction of a hygromycin resistance cassette. One of many complemented clones was selected for further study.

2.2. Western blotting

Lysates were prepared from exponential-phase aerobic cultures of *M. tuberculosis* H37Rv, *devR* mutant and complemented strains in Dubos Broth Base, in the absence of any antibiotics or in the presence of kanamycin (20 μg ml^{-1}) or kanamycin (20 μg ml^{-1}) and hygromycin (50 μg ml^{-1}), respectively. Lysates were electrophoresed on 15% sodium dodecyl sulfate–polyacrylamide gel and subjected to immunoblotting using 1:1000 dilution of high titer polyclonal anti-DevR sera as described [15].

2.3. In vitro morphology analysis

M. tuberculosis strains were cultured as described above, transferred to 15-ml centrifuge tubes (Corning, Corning, NY, USA) and maintained under sealed and static conditions for 9 days, mixed by inverting five or six times and photographed.

2.4. M. tuberculosis infection of human monocytic cells

Bacterial stocks for infection were cultured in MB 7H9 with 10% Middlebrook ADC enrichment and 0.2% glycerol and quantified by assessment of colony-forming units (CFU) of plated serial 10-fold bacterial dilutions.

Adherent and non-adherent cell populations from peripheral blood of healthy, tuberculin-positive individuals were separated as previously described in detail [16]. The adherent cells were found to be 99% positive using non-specific esterase staining and are referred to as human MN. The non-adherent cells collected by gentle rinsing represented peripheral blood lymphocytes (PBL). Triplicate 100-μl aliquots of MN suspension (i.e. 10^5 MN) were aliquoted into round-bottomed 96-well plates and incubated overnight at 37°C. Supernatants were aspirated from plates the next day and infection performed with a 1:1 infecting ratio of mycobacteria in Iscove's modified Dulbecco's medium (IMDM) with 30% fresh autologous serum as described [16]. Plates were returned to the 37°C incubator for 1 h, supernatants were aspirated and saved and cells rinsed three times with RPMI 1640 containing 10% fetal calf serum. Burden of intracellular bacteria was assessed immediately after rinsing of non-phagocytosed *M. tuberculosis* and after a further 4 and 7 days. MN were lysed and serial dilutions of each supernatant and lysate were plated onto MB 7Hl0/OADC as previously described [16]. Plates were incubated at 37°C and CFU per ml of lysate, which is equivalent to CFU per 10^6 initial MN, was determined.

2.5. Growth inhibition by PBL

PBL that had been recovered as described above were washed, resuspended in IMDM with 5% autologous serum. Following infection of MN as above, 1×10^6 PBL were added to each well containing infected MN. The resulting 10:1 PBL to MN ratio was selected to approximate that of peripheral blood mononuclear cells. CFU were then assessed on days 4 and 7 as described above.

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2.6. Scanning electron microscopy

Exponential-phase aerobic cultures of *M. tuberculosis* H37Rv, *devR* mutant and complemented strains were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde for 16 h at 4°C, dehydrated and coated with gold. The samples were scanned under Philips LEO435VP scanning electron microscope at 15 kV accelerating voltage.

2.7. Immunoelectron microscopy

Human MN were isolated and infected with *M. tuberculosis* strains essentially as described above. On days 4 and 7 post infection, aliquots of MN infected with *M. tuberculosis* H37Rv or H37Ra strains were gently aspirated from wells, pelleted and washed with phosphate-buffered saline (PBS) three times. The cells were then processed for electron microscopy as previously described [11]. For immunolabelling, polyclonal anti-DevR antibody (dilution 1:20) raised against purified DevR protein of *M. tuberculosis* was used.

2.8. In vivo infection of guinea pigs

Frozen stocks of *M. tuberculosis* strains maintained at −70°C were thawed, the cells were pelleted, washed once with PBS, pH 7.2, and resuspended in PBS as 1-ml aliquots. Ten albino, random-bred guinea pigs (five animals per group, mean weight 450 g, range 270–485 g) received s.c. injections of 0.1 ml of resuspended bacilli (7×10^6 CFU of *M. tuberculosis* H37Rv and 3.2×10^7 CFU of *devR* mutant). The *M. tuberculosis* inocula were coded and the entire experiment (weight measurement, visual assessment of disease, histopathology examination and CFU determination) was performed in an unbiased manner. Infected guinea pigs were killed 47 days post infection. Postmortem examination was carried out immediately. The extent of visible tuberculosis in the organs was scored as described [17]. The macroscopically normal animals were scored 0 and the maximum combined score for all organs was 100. Maximum possible scores for spleen, liver and lung were 40, 30 and 20, respectively and that for the site of inoculation and its draining lymph nodes was 10. The lungs with the trachea, liver and lymph nodes were removed to bottles containing 10% formalin for histopathology analysis. After routine processing, portions of the lung and liver (2 cm×2 cm thickness) were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The whole spleen was removed aseptically and placed in McCartney bottles for bacteriology. The spleen was minced, homogenized in 5 ml of saline (0.85% NaCl solution) and 0.1 ml of the homogenate was serially diluted in 0.9 ml saline to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A 10-μl sample of each dilution was plated in duplicate on LJ slants. The slants were incubated at 37°C and CFU were counted after 5 weeks.

2.9. Statistical analysis

For monocyte experiments, comparisons of intracellular burden of mycobacteria were assessed by paired Student's *t*-test. For guinea pig experiments, comparisons of postmortem scores and splenic burden of *M. tuberculosis* were assessed by non-parametric Mann–Whitney test. P value <0.05 was considered as the statistical significance level.

3. Results

3.1. Construction of devR mutant and complemented strains of M. tuberculosis

Plasmid pJQ200SK*devR::kan* was electroporated into *M. tuberculosis* and 25 transformants selected on MB 7H10 agar containing kanamycin (20 μg ml^{-1}) were analyzed; only six were positive by PCR for Km^R, signifying a high frequency of spontaneous Km^R (∼75%). All six clones were also positive for *sacB* by PCR indicating the involvement of a single crossover event in the generation of Km^R colonies. Three (of six) were positive by PCR for wild-type *devR* (513 bp) and *devR::kan* products (1.8 kb) suggesting that integration of the plasmid-borne *devR::kan* copy into the *M. tuberculosis* chromosome had occurred. This was confirmed by Southern hybridization with the *devR* probe (Fig. 1B, lane 1), in contrast to a single hybridization signal of 3.8 kb seen in the parental strain (Fig. 1B, lane 2). Identical results were obtained using the *devS* gene as probe. Hybridization with the Kan^R gene probe highlighted the ∼9.5-kb fragment containing the vector backbone resulting from a single crossover recombination event (Fig. 1B). The merodiploid strain (Dup *devR*) contained both the wild-type and the disrupted copies of the *devR* locus (Fig. 1B, lane 1). Out of 87 Km^R and Suc^R transformants obtained 18 were positive by PCR for the disrupted copy of *devR*. Allelic exchange was confirmed by Southern hybridization; a double crossover event led to the replacement of the wild-type *devR* gene (3.8-kb signal) by *devR::kan* (5.1-kb hybridization signal), the size increment of the mutant of 1.3 kb corresponding to the *kan* cassette inserted in the *devR* locus. Hybridization with the Kan^R gene probe confirmed the retention of the *kan* cassette-disrupted *devR* gene copy in the mutant strain (Fig. 1B, lane 3). Gene disruption and complementation with the wild-type gene was confirmed by PCR (Fig. 1C). A schematic representation of the *devR* locus in the wild-type and mutant strains is shown in Fig. 1A.

3.2. Immunoblot analysis

Wild-type DevR protein of 23 kDa was detected in lysates prepared from exponential-phase cultures of *M. tuberculosis* H37Rv while a protein of ∼45 kDa was de-

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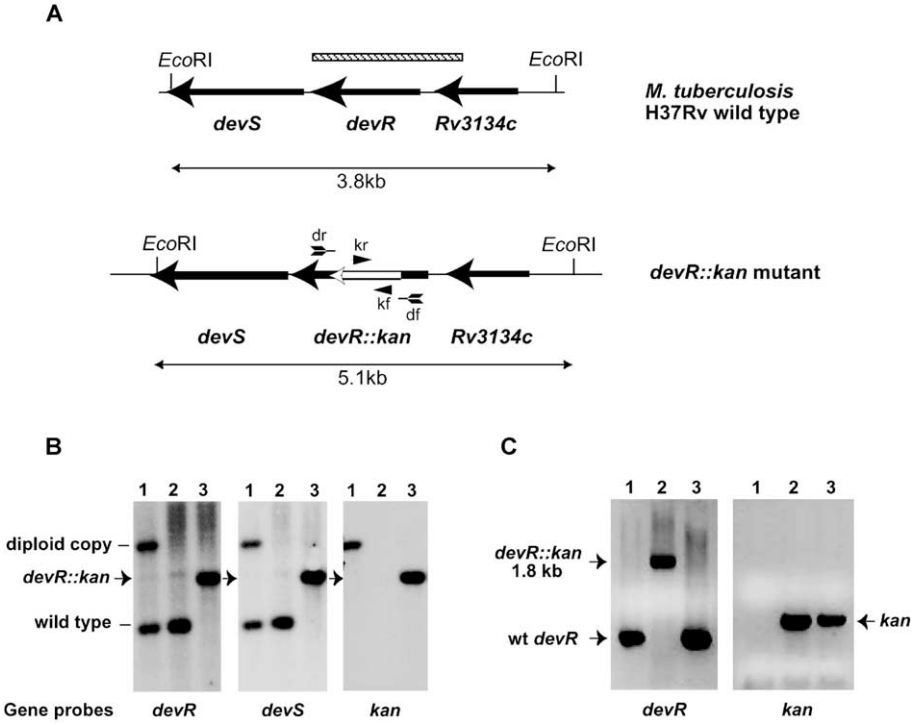

Fig. 1. Construction of *devR* mutant strain of *M. tuberculosis*. A: Schematic of the organization of the *devR* locus in wild-type and mutant strains (not to scale). The horizontal hatched bar indicates the region used in complementation. B: Southern hybridization. Genomic DNAs from *M. tuberculosis* H37Rv, *devR* mutant and Dup *devR* strains were digested with *Eco*RI and hybridized with ^{32}P-labelled DNA probes specific for *devR*, *devS* and *kan* genes. The disrupted copy of *devR* is indicated by an arrow. Lane 1, merodiploid strain of *M. tuberculosis*; lane 2, *M. tuberculosis* H37Rv; lane 3, *devR::kan* mutant. C: PCR analysis. *devR* sequences were amplified using primers df (5′-GGTGAGGCGGGTTCGGTCGC-3′) and dr (5′-CGCGGCTTGCGTCCGACGTTC-3′) to generate exclusively 513-bp and 1.8-kb products in wild-type and mutant strains, respectively. Primers kf (5′-TGGGAAGCCCGATGCGCCAG-3′) and kr (5′-CTCACCGAGGCAGTTCCATAGG-3′) were used to amplify the Kan^R gene (583-bp product). The 1.8-kb product was not amplified in the complemented strain because of competition from the shorter wild-type gene target. Lane 1, *M. tuberculosis* H37Rv; lane 2, *devR::kan* mutant; lane 3, *devR* complemented strain.

tected in lysates prepared from the mutant strain (Fig. 2A, lanes 1 and 2). DNA sequence analysis of the *devR* locus in the mutant strain indicated an in-frame insertion of the *kan* cassette encoding AphI after amino acid residue 145 of DevR. A fusion protein of 45 kDa containing the N-terminal domain of DevR (residues 1–145) and 261 amino acids encoding AphI was predicted by computer-aided translation of the sequenced region (Fig. 2B). This exactly matched the mass of the immunoreactive protein detected in the *devR* mutant strain (Fig. 2A, lane 2). We therefore believe that the *devR* mutant strain expresses a truncated DevR protein that lacks the C-terminal domain. Functional complementation was confirmed by the presence of both wild-type DevR and DevRN-Aph protein in lysates prepared from the complemented strain (Fig. 2A, lane 3).

3.3. *In vitro morphology and growth of* M. tuberculosis *strains*

devR mutant bacilli showed reduced cell-to-cell adherence and aggregate formation and were easily dispersible as compared to wild-type bacteria. This phenotypic change was reversed on complementation with a functional copy of *devR* (not shown) indicating the direct role of full-length DevR in this phenotype. The decrease in aggregative properties indicated that the bacterial surface was altered in mutant bacteria and could have a bearing on sensitivity to antibiotics. However, drug susceptibility assays revealed no differences in the sensitivities of wild-type and mutant bacilli to the commonly used antitubercular drugs including isoniazid, ethambutol, streptomycin, rifampicin and ofloxacin (not shown). The morphology of wild-type, mutant and complemented strains of bacilli was compared by scanning electron microscopy. All of them were found to be similar in shape and size (average 2–4 μm length, data not shown).

3.4. *Effect of* devR::kan *mutation on the intracellular replication of* M. tuberculosis

The effect of the *devR* mutation was assessed in a previously reported in vitro model of intracellular bacterial growth and lymphocyte-mediated restriction of intracellular growth [16]. MN purified from peripheral blood of 10 PPD-positive healthy subjects were infected with *M. tuberculosis* H37Rv and its *devR* mutant using a 1:1 bacteria-to-cell ratio. On days 0, 4 and 7, supernatants were removed and extracellular and intracellular CFU were sep-

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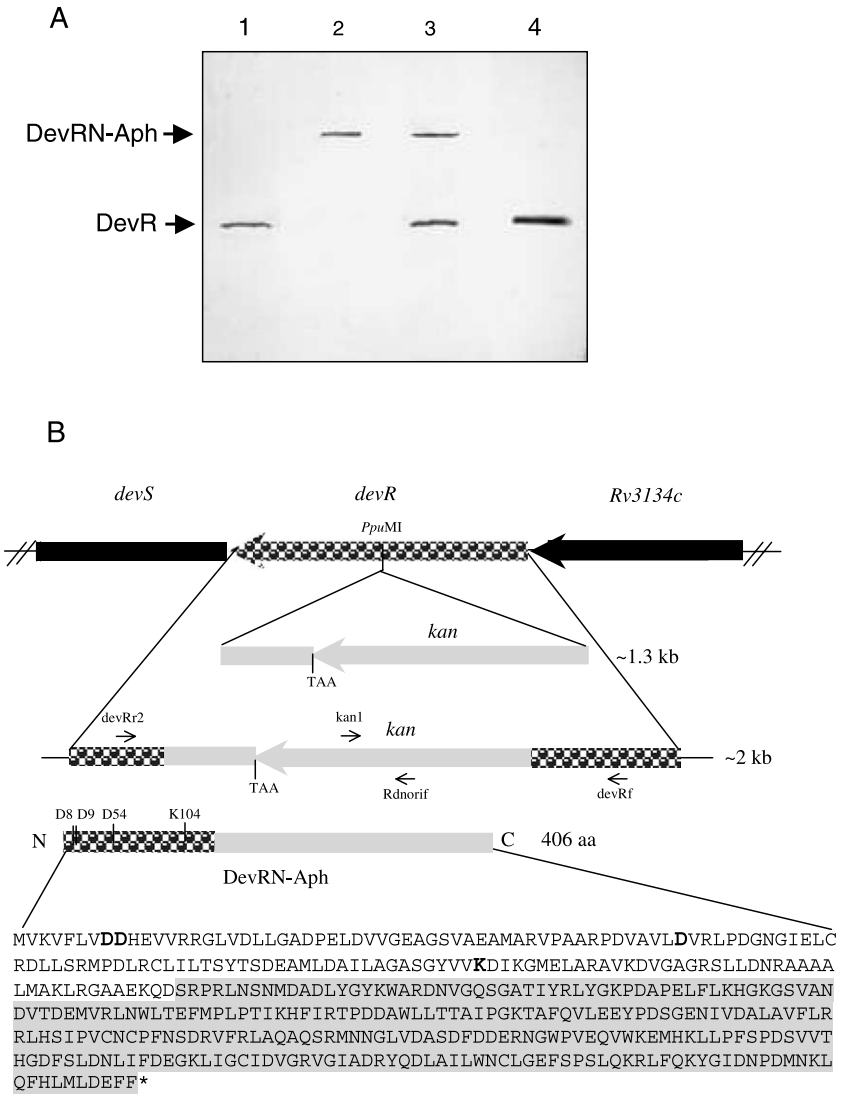

Fig. 2. Organization and expression of *devR* in *devR* mutant of *M. tuberculosis*. A: Western blot analysis of *M. tuberculosis* lysates. Lysates prepared from exponential-phase aerobic cultures were subjected to immunoblotting with anti-DevR antibody. Lane 1, *M. tuberculosis* H37Rv; lane 2, *devR::kan* mutant; lane 3, *devR* complemented strain; lane 4, purified DevR protein. B: The *kan* cassette (1.3 kb, in gray) inserted in the *devR* gene at the *Ppu*MI restriction site was fused in-frame with the N-terminal domain coding region of *devR* (in black ball pattern) to yield a 406-amino acid-long fusion protein that contained 145 amino acids of the N-terminal portion of DevR (residues critical for phosphorylation are indicated in bold) and 261 amino acids of AphI (lacking the first 8 amino acids). Thin arrows indicate the primers used to amplify the disrupted region in the mutant strain. The asterisk indicated the end of the Aph open reading frame.

arately determined by plating on MB 7H10 agar with OADC enrichment. Over the 7-day assay, H37Rv grew 14.4-fold (with a mean intracellular doubling time of 43.6 h) and yielded 1.32×10^6 bacilli while the *devR* mutant exhibited a similar 18.0-fold intracellular growth (corresponding to a mean intracellular doubling time of 40.2 h) and yielded 1.42×10^6 bacilli. The differences in initial and final CFU were not significant ($P=0.13$ and $P=0.69$ respectively by paired *t*-test). For both organisms, bacterial CFU within culture supernatants on day 4 and 7 was less than 1% of that of the cell pellet, indicating that release of organisms into the supernatant and/or extracellular growth was minimal (Fig. 3).

The two strains were also compared in their abilities to resist lymphocyte-mediated growth inhibition. For eight of the PPD-positive subjects studied above, growth of the parent and mutant strain within MN alone was compared to that observed following addition of autologous PBL to infected MN in a 10:1 PBL-to-MN ratio. As shown in Fig. 3, PBL mediated a mean reduction of 0.94 log in the intracellular growth of H37Rv over the course of the 7-day assay as compared to growth within MN alone. Similarly, PBL mediated a 1.04 log reduction in intracellular growth of *devR* mutant *M. tuberculosis* over the same time period. For both organisms, CFU within culture supernatants accounted for less than 2% of the decrease in intracellular organisms on day 4 and day 7, indicating that the observed decrease in intracellular CFU repre-

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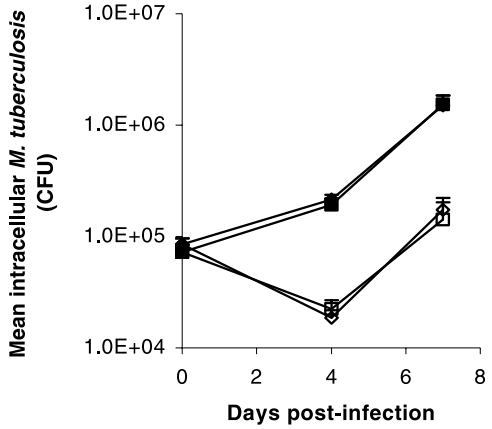

Fig. 3. Replication of *devR* mutant and wild-type strains of *M. tuberculosis* within human MN. Intracellular survival of *M. tuberculosis* H37Rv and *devR* mutant strains within MN or within MN plus non-adherent cells (NAC) from 10 (eight for MN+NAC) unrelated tuberculin-positive healthy human subjects. Data are presented as mean ± S.E.M. ♦, *M. tuberculosis* H37Rv in MN; ■, *M. tuberculosis devR* mutant in MN; ◇, *M. tuberculosis* H37Rv in MN+NAC; □, *M. tuberculosis devR* mutant in MN+NAC.

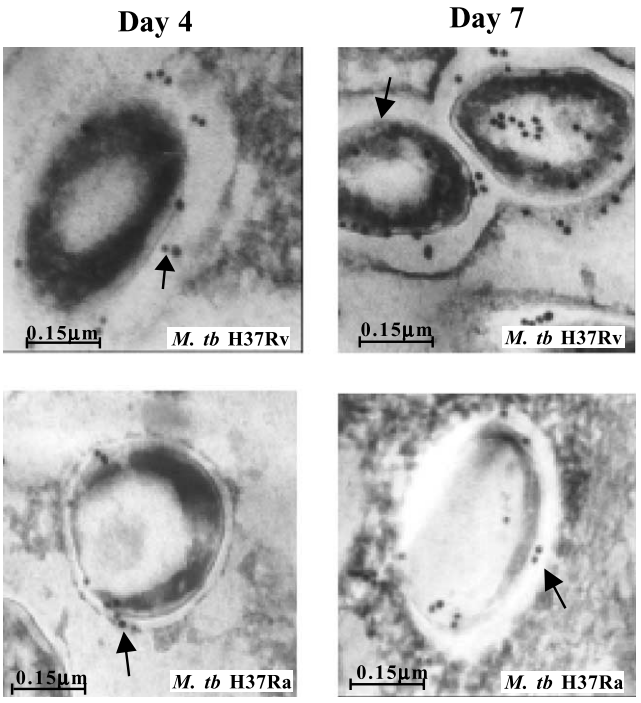

Fig. 4. Immunoelectron microscopy of MN infected with *M. tuberculosis* strains. Anti-DevR antibody and 15-nm gold particles were used to localize DevR protein (arrows) within vacuoles (vac) of human peripheral blood-derived MN infected with H37Rv and H37Ra strains of *M. tuberculosis* for the specified period.

sented inhibition of intracellular growth rather than release of viable organisms from MN. The differences in CFU on day 7 between the two strains in the presence of PBL again were not significant ($P = 0.42$ by paired *t*-test).

3.5. *DevR-DevS two-component system is not modulated within human MN*

DevR protein expression was monitored over a 7-day period in human MN infected with *M. tuberculosis* H37Rv (virulent) and H37Ra (avirulent) strains. DevR-specific immunostaining was detected within MN at both time points (Fig. 4). Labelling was observed at the surface, within the bacilli and also within the vacuolar space. The differential pattern of expression observed in virulent vs. avirulent strains in laboratory media-cultured bacteria was also noted within infected MN. The number of gold grains detected per *M. tuberculosis* H37Rv bacillus was 15 ± 3.1 and per H37Ra bacillus was 7 ± 1.2 on day 7 post infection. Therefore DevR expression did not appear to be preferentially modulated within MN in comparison to broth-cultured bacilli (15.6 ± 2.6 gold grains per H37Rv bacillus vs. 5.3 ± 1.4 gold grains per H37Ra bacillus).

3.6. M. tuberculosis devR *mutant strain is attenuated for virulence in guinea pigs*

The disease-causing capacity or 'virulence' of the *devR* mutant strain was studied in guinea pigs by assessing post-mortem scores, quantifying viable bacilli recoverable from spleen and the histology of affected organs. Animals were killed 47 days post infection. One animal (H37Rv group) that died a non-tuberculous death before the date of sacrifice was omitted from the analysis. The amount of visible tuberculosis in internal organs was scored immediately after sacrifice as described [17]. A heavy involvement of the lungs, liver, spleen and lymph node was noted in guinea pigs infected with *M. tuberculosis* H37Rv. The visual scores ranged between 43 and 93 (mean 77) and between 23 and 48 (mean 38.4) for guinea pigs infected with the parental and mutant strains, respectively, the difference being significant ($P < 0.05$, Table 1). The liver was the most affected organ and heavy invasion with numerous

Table 1
Characteristics of in vivo infection in guinea pigs infected with *M. tuberculosis* strains

Strain	Post-mortem visual scores[a]					Absolute viable count ($\log_{10}$ CFU spleen^{-1})
	Lung (20)	Liver (30)	Spleen (40)	Lymph node (10)	Total (100)	
H37Rv	13.75 ± 2.5	26.25 ± 7.5	30 ± 14.4	6.5 ± 14.4	77 ± 23.2	7.09 ± 0.83
devR mutant	6 ± 5.48	1.6 ± 3.58*	24 ± 13.42	6.8 ± 2.68	38.4 ± 12.64*	4.4 ± 1.21*

*$P < 0.05$ as derived by application of the Mann–Whitney test.
[a]Visual scores represent arithmetic mean ± S.D. from five and four animals for *M. tuberculosis devR* mutant and H37Rv strains respectively. The maximum score assigned for each organ is given within parentheses with a maximum body score of 100 as described [17].

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large tubercles and areas of necrosis was seen in guinea pigs infected with the parental strain. Spleen and lungs showed moderate invasion with numerous small tubercles. A considerably lower number of visible lesions was seen in the organs of guinea pigs infected with the mutant strain (Table 1).

Spleens were homogenized and serial dilutions were plated on LJ slants. A total of 7.09 ± 0.83 $\log_{10}$ CFU were isolated from spleens of animals infected with the parental strain vs. 4.4 ± 1.21 $\log_{10}$ CFU recovered from spleens of animals infected with the mutant strain, the difference being significant ($P < 0.05$, Table 1). *M. tuberculosis* DNA was isolated from spleen homogenates as described previously [18] and subjected to PCR analysis using *devR*- and *kan*^{r}-specific primers. Wild-type *devR* gene sequences (amplification product of 513 bp) were detected in homogenates of spleens isolated from guinea pigs infected with H37Rv but not mutant bacilli. Consistent with this finding, *kan*^{r} gene sequences were detected in homogenates of spleens from guinea pigs infected with mutant bacilli but not in H37Rv (Fig. 5A).

Liver, lung and lymph node autopsy specimens obtained from guinea pigs infected with *M. tuberculosis* H37Rv and *devR* mutant strains were examined for histological evidence of tuberculosis. Serial 5-μm sections (minimum three adjacent sections per animal) from these specimens were subjected to a semi-quantitative appraisal of the histological features (organ architecture, the percentage area occupied by the granuloma in the section and the percentage of the major cellular components within the granuloma) as described previously [19]. Liver sections from three of five guinea pigs infected with the mutant strain had normal architecture and did not show any granuloma or the presence of inflammatory cell infiltrates. In the remaining two animals infected with the mutant strain, minimal, well-organized, non-necrotic epithelioid cell granuloma was observed. Liver sections from all four guinea pigs infected with the parental strain showed the presence of well-formed granuloma that consisted of epithelioid cells and lymphocytes. Other cell types were absent. In one animal, the granuloma was extensive (65%) and was accompanied by the partial destruction of organ architecture. Compared to the liver, much more extensive involvement of the lung was noted. The lung architecture in all the guinea pigs infected with the mutant strain was normal. Although all the animals showed the presence of granuloma, it was minimal and consisted of both lymphocytes and macrophages. Giant cells and other cells or necrosis were not seen in any of the lungs. In animals infected with the parental strain, the lung from one guinea pig was completely destroyed and in the remaining three it was partially damaged. The granuloma ranged from 40% to 85% and varied from being predominantly lymphocytic to mainly histiocytic (Fig. 5B). Minimal fibrinoid necrosis was observed in one animal. On acid-fast staining, occasional bacilli were seen in three of the lung specimens and

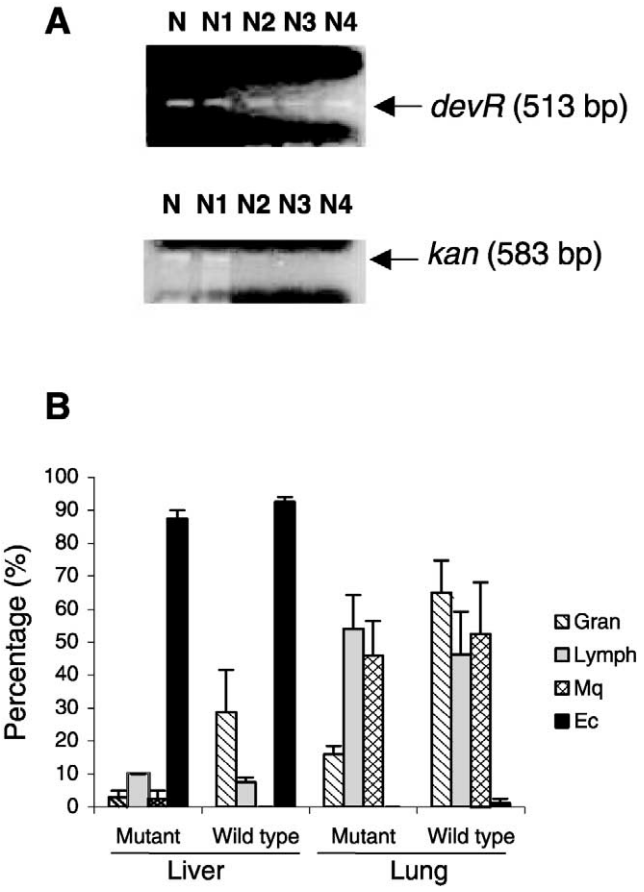

Fig. 5. A: Detection of *M. tuberculosis* DNA in spleens of guinea pigs infected with wild-type and *devR* mutant bacteria. Spleen homogenates were analyzed by PCR for the presence of DNA sequences specific for the infecting organisms. The 513-bp product observed with *devR*-specific primers was specific for wild-type organisms (top panel) and the 583-bp product with *kan*^{r} primers was specific for *devR* mutant bacilli (bottom panel). N, N1, N2, N3 and N4 refer to neat and 10-fold dilutions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}), respectively of spleen homogenates used for DNA isolation. The sequences of the amplification primers are provided in the legend to Fig. 1C. B: Characteristics of liver and lung granuloma in guinea pigs infected with wild-type and *devR* mutant strains of *M. tuberculosis*. Hematoxylin and eosin-stained sections were examined and a semi-quantitative appraisal of the histological features was made. The mean % granuloma (Gran) and % lymphocytes (Lymph), macrophages (Mq) and epithelioid (Ec) cells in granuloma observed in three adjacent sections of liver and lungs of animals in each group are plotted as mean ± S.E.M.

these were present mainly amongst the collections of macrophages. In two guinea pigs infected with the *devR* mutant strain and in one infected with the parental strain, the inguinal lymph node was enlarged. On histological examination, it was noted that all three nodes were replaced completely by a centrally caseating, mycobacterial granuloma.

4. Discussion

In the mutant strain described here, the *devR* gene was disrupted by insertion of a *kan* cassette, which led to the

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expression of a fusion protein comprising the N-terminal domain of DevR and AphI. Furthermore, since the upstream promoter elements were intact, the expression of the flanking genes, *Rv3134c* and *devS*, encoding a protein of the Usp family and DevS sensor, respectively, was expected to be unhindered. This supposition was experimentally verified; *Rv3134c* and *devS* genes were expressed in the mutant bacilli [5]. We therefore believe the phenotypic properties of the mutant strain to be a consequence of the absence of full-length functional DevR protein. Since the C-terminal domain containing the DNA binding activity is absent in the mutant bacilli, a crucial role for it in DevR function is implied.

Our findings support the notion that DevR function is linked to physiological processes inherent in prolonged infection and less relevant to early events in infection. Firstly, the *devR* mutant and parental strains replicated at equivalent rates within human MN alone or within MN supplemented with PBL. Secondly, the expression of DevR protein was not upregulated upon entry of *M. tuberculosis* into MN in comparison to broth-cultured bacilli. These results collectively suggest that the conditions that presumably activate DevR-DevS do not occur in response to phagocytic or other early events. Thirdly, the mutant strain was attenuated in virulence at the end of 7 weeks post infection; significantly fewer gross lesions and organ pathology and a nearly 1000-fold lower bacterial load were observed in guinea pigs infected with the *devR* mutant strain compared to guinea pigs infected with the parental strain. The preponderance of epithelioid cells over macrophages and lymphocytes in liver as compared to lung was suggestive of a good immune response and more advanced resolution of granuloma in the former. Liver from guinea pigs infected with the mutant strain showed less granuloma formation that may be a consequence of rapid clearing of the mutant bacilli from the liver. This assumption is consistent with the lower liver weight ratio observed with this strain. Thus the mutant strain failed to cause severe progressive disease and pathology under the present experimental conditions as compared to the wild-type strain.

It was recently reported that a *devR* mutant of *M. tuberculosis* is hypervirulent [20] which is inconsistent with our results. There are some possible explanations for this discrepancy. First, the mutant strains were different, as we used a strain in which the N-terminal domain of DevR was expressed whereas they used a strain deleted of the entire *devR* gene. Second, we assessed our mutant in immunocompetent guinea pigs injected by the s.c. route and measured gross lesions, lung and liver pathology and splenic bacterial load, while Parish et al. [20] used i.v. inoculation of SCID mice and immunocompetent mice to assay survival and tissue bacterial loads, respectively. The initial distribution of the inoculated bacteria within the body of the animal varies according to the route of introduction. In the s.c. route the bulk of the inoculum remains localized at the site of the injection but there is also some dissemination to the local lymphatic tissue and, if blood vessels are punctured, into the blood; while in the i.v. route the whole inoculum is distributed through the circulation and the bulk of the bacilli are removed rapidly from the bloodstream and found in the liver and spleen [21]. The aspect of the s.c. route that makes it superior to other (i.m., i.p., i.v.) routes of infection is that it is the only alternative to the respiratory route which mimics the local deposition of bacilli in a defined anatomical site, followed by local growth, progressive dissemination through the lymphatics into the circulation and eventually the hematogenous seeding of other organs (lung, spleen, liver, etc.) to multiply and produce gross disease in lungs, liver and spleen. In contrast the i.v. route directly places the tubercle bacilli in the bloodstream and does not evaluate the potential of tubercle bacilli to spread from inoculation site via the bloodstream (D. McMurray, personal communication). Further, conventionally because of difficulty in accessibility of veins, guinea pigs are not infected by the i.v. route. Although the s.c. route requires a much higher bacillary load (10–100-fold) for challenge it essentially produces disease similar to the widely accepted aerosol route [22].

A valuable clue to the stage(s) in pathogenesis of DevR involvement was obtained when *devR-devS* of *M. tuberculosis* was found to be induced under hypoxia [10], an environmental condition thought to prevail within inflammatory and necrotic lesions [23,24]. In *M. bovis* BCG, a *dosR* (*devR*) mutant showed greatly reduced survival under hypoxic conditions in an in vitro model and DosR was suggested to be a key regulator of hypoxia-induced mycobacterial dormancy response [25]. Tubercle bacilli likely reside indefinitely in a stationary phase-like 'persistent' state as a consequence of successful adaptation to hypoxia and/or nutrient starvation within granulomas. The DevR-DevS system has been recently confirmed to be an authentic signal transduction system by demonstration of the phosphorylation activities characteristic of histidine kinase and response regulator proteins using purified DevR and DevS proteins of *M. tuberculosis* [5]. We propose that DevR-DevS mediates bacterial adaptation to hypoxia through a phosphorylation cascade and could well be a key regulatory link between oxygen limitation and the initiation and/or maintenance of the adaptive response to hypoxia. Additional experiments are necessary to dissect the hypoxia response and identify its various components.

5. Note added in revision

While this article was under revision Park et al. [Mol. Microbiol. 48 (2003) 833–343] reported Rv3133c/DosR (DevR) to be a transcriptional regulator of the hypoxia response of *M. tuberculosis*.

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Acknowledgements

The generous gift of pJQ200SK by Dr. M.F. Hynes, Canada, is gratefully acknowledged. We are grateful to Illah Joshi for her assistance with experiments. The assistance of Dr. M. Naseema and M. Asokan of TRC with the animal experiments is acknowledged. The technical assistance of S. Jaya Gopinath, S. Nambirajan and K. Chandran of TRC is also acknowledged. R.F.S. is grateful to the NIH for financial assistance through NIH Grant HL-59858. R.F.S. is the recipient of a Parker B. Francis Fellowship in Pulmonary Research sponsored by the Francis Families Foundation. Monocyte infection assays at Cleveland were according to protocols approved by the IRB of Case Western Reserve University and University Hospitals of Cleveland. V.M. and D.S. are grateful to CSIR for Senior Research Fellowships. J.S.T. is grateful to the Department of Biotechnology, Government of India, for financial support throughout the study.

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