Involvement of the *fadD33* gene in the growth of *Mycobacterium tuberculosis* in the liver of BALB/c mice

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The potential pathogenic role of *Mycobacterium tuberculosis* H37Rv *fadD33*, a gene encoding an acyl-CoA synthase that is underexpressed in the attenuated strain H37Ra, was investigated. In a first approach, *fadD33* was cloned and expressed in strain H37Ra to restore gene expression and *fadD33*-complemented bacteria were used to investigate whether *fadD33* might confer any growth advantage to *M. tuberculosis* H37Ra in an infection model of BALB/c mice. No differences were found in the growth rates of *M. tuberculosis* H37Rv, H37Ra and *fadD33*-complemented H37Ra in the lungs and spleen. In contrast, in the liver, where the attenuated strain H37Ra showed impaired growth compared to the virulent strain H37Rv, complementation of the attenuated strain H37Ra with *fadD33* restored bacterial replication. In a further approach, the *fadD33* gene of strain H37Rv was disrupted by allelic exchange mutagenesis and the virulence of the mutant strain was tested by mouse infection. It was found that disruption of *fadD33* decreased *M. tuberculosis* H37Rv growth in the liver, but not in the lungs or spleen, and complementation of the *fadD33*-disrupted mutant with *fadD33* restored bacterial replication in the liver, but did not affect replication in the lungs and spleen. These findings suggest that *fadD33* plays a role in *M. tuberculosis* virulence by supporting bacterial growth in the liver.

Keywords: virulence genes, experimental infection

INTRODUCTION

Although tuberculosis remains one of the leading causes of death worldwide, the mechanisms by which *Mycobacterium tuberculosis* establishes progressive disease are not well understood. Identification of the virulence factors of *M. tuberculosis* is of fundamental importance for the development of new vaccines and drugs against this pathogen.

A widely used experimental model for the investigation of *M. tuberculosis* virulence factors at the gene level is represented by the virulent strain H37Rv and the attenuated mutant H37Ra, originally derived from the classical *M. tuberculosis* strain H37 (Steenken *et al.*, 1934). A number of extensive studies have been tried so

far to identify the genetic basis for the attenuation of *M. tuberculosis* H37Ra. For example, by transforming *M. tuberculosis* H37Ra with a cosmid library of *M. tuberculosis* H37Rv, Pascopella *et al.* (1994) identified a locus that enhanced growth and survival of *M. tuberculosis* H37Rv in the spleens of infected mice. Furthermore, comparison of *M. tuberculosis* H37Rv with *M. tuberculosis* H37Ra revealed differences in gene expression (Kinger & Tyagi, 1993; Rindi *et al.*, 1999; Rivera-Marrero *et al.*, 1998), transposition of insertion elements (Lari *et al.*, 1999, 2001) and genomic polymorphisms and deletions (Brosch *et al.*, 1999), but the reason(s) for the decreased virulence of *M. tuberculosis* H37Ra have not been determined.

By comparing gene expression of the H37Rv and H37Ra strains by mRNA differential display, Rindi *et al.* (1999) reported a number of genes that appear to be down-regulated in strain H37Ra. Sequences of these genes

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Abbreviation: Kan^R, kanamycin-resistance.

were found only in species belonging to the *M. tuberculosis* complex, i.e. *M. tuberculosis*, *Mycobacterium bovis*, including the BCG strain, and *Mycobacterium microti*, thus indicating their association with the most virulent mycobacteria (Rindi *et al.*, 2001). One of these genes, *fadD33* (also called *Rv1345*), is one of 36 homologues of the *fadD* gene of *Escherichia coli* identified in the *M. tuberculosis* genome (Cole *et al.*, 1998). In *E. coli*, the *fadD* gene product is an acyl-CoA synthase responsible for the activation of free fatty acids to acyl-CoA thioesters, which represents the first step of fatty acid degradation (Clark & Cronan, 1996; Nunn, 1987). Predictively, mycobacterial *fadD33* encodes an acyl-CoA synthase as well, but the precise biochemical function of FadD33 is not defined at present.

The purpose of the study presented here was to address the question of whether *fadD33* plays a role in *M. tuberculosis* infection. For this purpose, we cloned and expressed the *fadD33* gene of *M. tuberculosis* H37Rv in the attenuated strain H37Ra to restore gene expression; furthermore, we constructed a mutant of strain H37Rv with a disrupted *fadD33* gene. Virulence of *fadD33*-complemented *M. tuberculosis* H37Ra and *fadD33*-disrupted *M. tuberculosis* H37Rv was then tested by experimental mouse infection.

METHODS

Bacterial strains and growth conditions. *M. tuberculosis* H37Rv and H37Ra were from a collection maintained at our Department. The IS6110-RFLP patterns of the strains have been reported previously (Lari *et al.*, 1999, 2001) and correspond to genotypes Rv4 and Ra1 of the H37 variants defined by Bifani *et al.* (2000). The H37Rv and H37Ra strains were grown in liquid Middlebrook 7H9 medium (Difco Laboratories) supplemented with 0.25% Tween 80 (Difco) and albumin–dextrose complex (ADC) [0.5% albumin, fraction V (Sigma), 0.085% NaCl, 0.2% glucose]. Middlebrook 7H10 plates supplemented with 10% OADC enrichment (Becton Dickinson) were used for colony isolation. *M. tuberculosis* H37Rv and H37Ra wild-type strains and recombinant and/or mutant strains used in the present investigation, i.e. H37Ra-*fadD33*, H37Rv-*fadD33*::Kan and *fadD33*-complemented H37Rv-*fadD33*::Kan (see below), grew with similar kinetics in axenic cultures, as determined by the radiometric BACTEC TB 460 system (Becton Dickinson) and by counts of colony-forming units (c.f.u.s) on 7H10 agar plates.

E. coli strains XL-1 Blue, JM109 and DH5 α (Stratagene) were used for recombinant DNA studies and plasmid propagation. These strains were grown on solid or in liquid Luria–Bertani (LB) medium (Sigma). When required, antibiotics were included in the media at the following concentrations: 100 μ g hygromycin ml⁻¹, 20 μ g kanamycin ml⁻¹ and 5 μ g gentamicin ml⁻¹ for mycobacteria, and 100 μ g hygromycin ml⁻¹, 50 μ g kanamycin ml⁻¹ and 20 μ g gentamicin ml⁻¹ for *E. coli*.

Recombinant DNA techniques. Extraction of mycobacterial genomic DNA, molecular cloning and restriction endonuclease digestions were performed by standard techniques (Sambrook *et al.*, 1989). Cloning vectors used were pBluescript KS(+) (Stratagene), pROLHYG, pMY9 and pPR27 (Pelicic *et al.*, 1997). Plasmid pROLHYG was obtained from the shuttle plasmid pHYG16R1 (Garbe *et al.*, 1994) by the addition of the

pBluescript-II-SK polylinker in the *KpnI* site. Plasmid pMY9 was constructed by cloning the Tn903 *aph* gene conferring kanamycin-resistance (Kan^R) into *EcoRI*-digested pUC8 (Yanisch-Perron *et al.*, 1985). The Kan^R cassette was amplified by PCR from pMD31 (Donnelly-Wu *et al.*, 1993) using *EcoRI*-tailed primers (5'-GCGAATTCCTTTTCTGTGACTGGTGA-3' and 5'-CGGAATTCTCTCAGAATGACTTGGTTG-3') and digested with *EcoRI*. Restriction endonucleases and other enzymes (Roche Molecular Biochemicals) were used according to the manufacturers' instructions.

Cloning of *fadD33* and complementation of *M. tuberculosis* H37Ra and the H37Rv-*fadD33*::Kan mutant. A 1.809 kbp fragment of *M. tuberculosis* H37Rv chromosomal DNA, including the full-length *fadD33* gene (GenBank accession no. Q11015; sequence positions 1509281–1510843), was amplified by PCR. Primers PKup (5'-ACGCTGCTGAGGATCCTG-3') and PKlow (5'-CAGCAGCTCCAGCCTAGG-3'), annealing to bases 217–199 upstream (in order to include the promoter) and bases 9–26 downstream of the *fadD33* reading frame, respectively, were used. The primers had a base mutation (C→G and G→T, respectively) to create *Bam*HI restriction sites. The PCR-generated fragment was cloned in *E. coli* XL-1 Blue into the *E. coli*–*Mycobacterium* shuttle vector pROLHYG, encoding hygromycin resistance, which had been digested with *Bam*HI (Boehringer), according to standard protocols (Sambrook *et al.*, 1989). The resulting plasmid (pROLHYG-*fadD33*) was propagated in *E. coli* XL-1 Blue after selection on LB agar containing 100 μ g hygromycin ml⁻¹ (Sigma). The integrity of the coding region was verified by sequencing the inserted DNA fragment using an automated apparatus (ALFexpress DNA sequencer; Pharmacia Biotech), using the Cy5 Thermo Sequenase dye Terminator Kit (Pharmacia). The recombinant pROLHYG-*fadD33* was then used to transform by electroporation *M. tuberculosis* H37Ra and the H37Rv-*fadD33*::Kan mutant (see below). Transformants were selected by growth at 37 °C on Middlebrook 7H10 agar containing 100 μ g hygromycin B ml⁻¹.

Construction of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant. A 2.971 kbp *Xba*I–*Clal* fragment containing *fadD33* was cloned in *E. coli* into pBluescript KS(+) by standard methods (Sambrook *et al.*, 1989) and a 275 bp intragenic fragment of *fadD33* was removed by *EcoRI* digestion. The *EcoRI* 1 kbp Kan^R cassette from pMY9 was inserted at the *EcoRI* site present in *fadD33*. The resulting 3.9 kbp *Xba*I–*Sfi*I fragment, which contained 1.7 kbp of *fadD33* left-flanking DNA and 1.2 kbp of *fadD33* right-flanking sequenced around a central Kan^R gene, was excised and blunt-end cloned into pPR27, a mycobacterial suicide vector carrying gentamicin resistance, sucrose sensitivity and an origin of replication thermosensitive in mycobacteria, to yield pPR27-*fadD33*::Kan. Approximately 1 μ g pPR27-*fadD33*::Kan was introduced into electrocompetent *M. tuberculosis* H37Rv. Five millilitres of 7H9/ADC broth without antibiotics was added immediately, and the bacteria were incubated at 32 °C for 24 h. Transformants were selected at 32 °C on 7H10/kanamycin and were grown in 7H9/kanamycin at 32 °C until saturation. The cultures were then plated at 39 °C onto 7H10/kanamycin with 2% sucrose. PCR analysis was performed to screen for disruption of *fadD33*. In particular, primers [PKa (5'-ATGACCGCGGCACCGAAGCTT-3') and PKb (5'-GATCCAGGCGACACGAAGAC-3')] that flanked the deletion site were used, producing a 758 bp fragment in the wild-type and a 1483 bp fragment in the deletion strain. The PCR mix contained 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 250 ng each primer, 0.2 mM each dNTP, 1.25 U *Taq* polymerase (Dynazyme) and

10 ng DNA per 50 µl of reaction mixture. PCR amplification was performed with an OmniGene temperature cycler (Hybaid) set for one cycle of 3 min at 94 °C, 25 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, with a final cycle for 4 min at 72 °C. Aliquots (10 µl) of PCR products were analysed by 2% agarose gel electrophoresis.

Electrotransformation. *M. tuberculosis* H37Rv and H37Ra were prepared for electroporation as described by Pelicic *et al.* (1997), with minor modifications. Briefly, cells were grown in 200 ml Middlebrook 7H9/ADC/Tween 80 for 3 weeks at 37 °C, washed three times in 10% (v/v) glycerol, resuspended in 1 ml of 10% glycerol and stored at -80 °C until needed. Aliquots (100 µl) of competent cells were mixed with approximately 1 µg recombinant DNA in a Bio-Rad cuvette with a 0.2 cm gap width and electrotransformed with a Bio-Rad Gene Pulser and Pulse Controller set at 25 µF, 200 Ω and 2.5 kV.

Gene expression analysis by RT-PCR. Total RNA was extracted from the pellet of mycobacterial cells growing in liquid Middlebrook medium essentially as described by Chomczynski & Sacchi (1987) with minor modifications (Rindi *et al.*, 1998). RNA reverse transcription was carried out by using oligo(dT)-primed reverse transcriptase (RT), as described previously (Rindi *et al.*, 1998). Briefly, a 1 µg sample of bacterial RNA, dissolved in diethyl pyrocarbonate (DEPC)-treated water, was heated at 65 °C for 15 min and then cooled on ice before cDNA synthesis. Oligo(dT)₁₅ (Pharmacia Biotech) was used to initiate the first strand cDNA synthesis, using 200 U M-MLV RT (Gibco) in a buffer containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 0.5 mM each dNTP, 20 U RNase inhibitor and 25 pmol primer for 1 h at 37 °C in a total volume of 20 µl. The reaction was stopped by incubation at 95 °C for 5 min. A 175 bp *fadD33*-specific target sequence of the generated cDNAs was PCR-amplified using oligonucleotide primers PK1 (5'-TGC-TTGCCGACCGTGTCTATC-3') and PK2 (5'-CCCAGGTA-ACCCGCCATCAT-3'), which anneal to bases 980-999 (upstream) and bases 1135-1154 (downstream), respectively, of the *fadD33* reading frame. Briefly, PCR was performed in 0.5 ml microcentrifuge reaction tubes in a final volume of 50 µl containing 10 mM Tris/HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 250 ng each primer, 0.2 mM each dNTP and 1.25 U *Taq* polymerase (Dynazyme). PCR amplification was performed with an OmniGene temperature cycler (Hybaid) set for one cycle of 3 min at 94 °C, 25 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C, with a final cycle of 4 min at 72 °C. For *Rv0441c* expression studies, a 225 bp specific target of the *M. tuberculosis* *Rv0441c* gene encoding the 65 kDa antigen (GenBank accession no. M15467) was PCR-amplified using primers 65K1 (5'-CGGTTTCGACAAG-GGCTACATC-3') and 65K2 (5'-GCGGATCTTGTTGACG-ACCAG-3'), as described previously (Rindi *et al.*, 1998). Aliquots (10 µl) of the PCR products were analysed by 2% agarose gel electrophoresis.

Infection of mice. BALB/c male mice (Calco, Como, Italy) were infected intravenously with 0.2 ml aliquots of mycobacterial suspensions containing approximately 1×10^5 c.f.u. (0.2 ml)⁻¹. At each time point, mice were killed and the number of c.f.u.s in the spleen, lung and liver were determined. Organs were aseptically removed and homogenized in Middlebrook 7H9 broth. To enumerate c.f.u. values, 0.5 ml aliquots of 10-fold serial dilutions of homogenates were plated onto Middlebrook 7H10 agar medium and colonies were counted after 3 weeks incubation at 37 °C in a humidified 5% CO₂ atmosphere. The number of c.f.u.s per entire organ was

reported as the log₁₀ value. The significance of the difference in the mean number of c.f.u.s per organ was calculated by the ANOVA test according to the INSTAT software package (GraphPad); all *P* values less than 0.05 were considered to indicate statistical significance.

RESULTS

Cloning of *fadD33*

A 1809 bp DNA fragment, including the 1563 bp *fadD33* gene and its promoter, was PCR-amplified from *M. tuberculosis* H37Rv genomic DNA by using a pair of specific primers. The amplified product was cloned in *E. coli* XL-1 Blue into the *E. coli*-*Mycobacterium* shuttle expression vector pROLHYG and 60 colonies were examined for the presence of the insert by restriction analysis. Six *E. coli* transformants, which harboured pROLHYG containing the insert of the expected length, were sequenced. Five of these showed nucleotide sequences with 1-3 single base mutations determining an incorrect *fadD33* gene product; one transformant, selected for the present study, showed a nucleotide sequence encoding the expected product of *fadD33*, although a silent single base mutation (C→T) was found at gene position 430.

Complementation of *M. tuberculosis* H37Ra

The recombinant shuttle plasmid pROLHYG-*fadD33* was electroporated into *M. tuberculosis* H37Ra and the expression of *fadD33* in these cells was analysed by RT-PCR. In particular, total DNase-treated RNA from *M. tuberculosis* H37Ra(pROLHYG-*fadD33*), *M. tuberculosis* H37Ra(pROLHYG), *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv was transcribed into cDNA by using oligo(dT)-primed reverse transcriptase. The reaction products were then probed by PCR employing primers specific for the *fadD33* gene and for a constitutively expressed gene, i.e. *Rv0441c*, which encodes the 65 kDa antigen (Shinnick *et al.*, 1987). The identity of the amplified cDNA obtained by using primers PK1 and PK2 was verified by sequencing. As shown in Fig. 1, *fadD33*-specific bands were detected for all of the mRNA transcripts; the signal for the *fadD33* messenger from strains H37Ra and H37Ra(pROLHYG) was markedly weaker than that obtained from *M. tuberculosis* H37Ra(pROLHYG-*fadD33*) and wild-type H37Rv, while the RNA transcripts for the constitutively expressed 65 kDa antigen gene yielded bands of equivalent intensity in all of the strains.

Mouse infection by *fadD33*-complemented *M. tuberculosis* H37Ra

As a first attempt to examine the role of *fadD33* on *in vivo* growth and survival, BALB/c mice were infected intravenously with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *fadD33*-complemented H37Ra. The mice were killed at 1, 21 and 42 days post-infection, and the number of c.f.u.s in the lungs, spleen and liver were

Fig. 1. Expression of *fadD33* in *fadD33*-complemented *M. tuberculosis* H37Ra. The image shows an ethidium-bromide-stained gel of the RT-PCR products, separated by 2% agarose gel electrophoresis, which were obtained from RNA extracted from *M. tuberculosis* H37Ra, H37Rv, H37Ra electroporated with pROLHYG (H37Ra-pROL) and H37Ra electroporated with pROLHYG-*fadD33* (H37Ra-*fadD33*) and probed for cDNA of *fadD33* and *Rv0441c* (indicated at the bottom). Lanes also include a molecular mass marker (100 bp ladder), PCR carried out in the absence of cDNA (H₂O), RT-PCR products of RNA from *M. tuberculosis* H37Ra-*fadD33* (RNA H37Ra-*fadD33*) and PCR products of DNA from *M. tuberculosis* H37Rv (DNA H37Rv).

determined (Fig. 2). As to virulent *M. tuberculosis* H37Rv, the number of c.f.u.s increased by approximately 1 log₁₀ in the spleen, 1.5 log₁₀ in the lung and 0.5 log₁₀ in the liver during the first 3 weeks of infection. Thereafter, the bacillary load stabilized at approximately 10^{5.5} c.f.u.s in the three organs. In the lungs and spleen, the log₁₀ c.f.u. counts of strain H37Ra, as well as those of the *fadD33*-complemented H37Ra, were comparable to H37Rv at any tested time, which might indicate that the H37Rv strain employed in our experiments is not as virulent as expected. In the liver, however, the log₁₀ c.f.u. counts for strain H37Ra were significantly lower than those for strain H37Rv at 21 and 42 days post-infection ($P < 0.001$), and those of the *fadD33*-complemented H37Ra strain were significantly higher than those of strain H37Ra ($P < 0.01$), and not statistically different from strain H37Rv. No animal deaths were recorded during the whole experimental period. These results demonstrate that complementation of strain H37Ra with *fadD33* conferred a growth advantage to the attenuated strain H37Ra in the liver.

Construction of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant

A fragment of the *fadD33* gene of *M. tuberculosis* H37Rv was replaced with a Kan^R gene by allelic exchange using *ts-sacB* counterselection technology

Fig. 2. Experimental infection of BALB/c mice with *M. tuberculosis* H37Rv (■), *M. tuberculosis* H37Ra (●) and *M. tuberculosis* H37Ra complemented with *fadD33* (▲). The data are expressed as the geometric means $\pm$ SD of the log₁₀ c.f.u. counts obtained from four mice per group per time point.

(Pelicic *et al.*, 1997). The recombinant plasmid pPR27-*fadD33*::Kan was introduced into *M. tuberculosis* H37Rv by electroporation, and transformants were selected at 32 °C on 7H10/kanamycin. Several transformants were grown in liquid culture and then plated at 39 °C onto 7H10/kanamycin containing 2% sucrose. Various colonies were grown in 7H9 medium and genomic DNA was extracted and analysed by PCR using a pair of primers flanking the deletion site (Fig. 3). *M. tuberculosis* H37Rv DNA, which was included as a control, showed one amplification band of 753 bp in size. As expected for allelic exchange mutants, all of the clones presented a single fragment approximately 700 bp longer than that in the wild-type strain. This 700 bp increase corresponded to the size of the Kan^R gene

Fig. 3. *fadD33* region in *M. tuberculosis* allelic exchange mutants (H37Rv-*fadD33*::Kan). (Left panel) PCR products obtained from DNA of four clones of H37Rv-*fadD33*::Kan mutants (lanes 1–4) and wild-type H37Rv. Lanes also include a molecular mass marker (100 bp ladder) and PCR carried out in the absence of cDNA (H₂O). (Right panel) The *fadD33* region of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant and *M. tuberculosis* H37Rv is outlined. Dark-grey, light-grey and solid bars represent the bacterial chromosome, the *fadD33* gene and the Kan^R box, respectively. The arrows indicate the PCR primer positions; the expected amplicon sizes are also shown.

(1 kbp) that was inserted in place of a 275 bp fragment of the *fadD33* gene. The wild-type and mutant strains of *M. tuberculosis* H37Rv showed similar colony morphology, growth characteristics and drug susceptibility profiles (data not shown).

As a control, the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant was complemented with *fadD33* by electroporation with the recombinant plasmid pROLHYG-*fadD33* (as described above). The expression of *fadD33* in these cells was analysed by an oligo(dT)-primed RT-PCR assay (Rindi *et al.*, 1998). For this purpose, total DNase-treated RNA from *M. tuberculosis* H37Rv-*fadD33*::Kan, *M. tuberculosis* H37Rv-*fadD33*::Kan(pROLHYG-*fadD33*) and *M. tuberculosis* H37Rv was transcribed into cDNA by using oligo(dT)-primed reverse transcriptase. The reaction products were then probed by PCR employing primers specific for the *fadD33* and *Rv0441c* genes. Sequence-verified *fadD33*-specific bands were detected for the mRNA transcripts of *M. tuberculosis* H37Rv-*fadD33*::Kan(pROLHYG-*fadD33*) and *M. tuberculosis* H37Rv, but not from *M. tuberculosis* H37Rv-*fadD33*::Kan; the RNA transcripts for *Rv0441c* yielded the expected bands in all of the strains (Fig. 4).

Mouse infection by the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant

BALB/c mice were infected intravenously with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Rv-*fadD33*::Kan mutant and *M. tuberculosis* H37Rv-*fadD33*::Kan(pROLHYG-*fadD33*), and the number of c.f.u.s in the lungs, spleen and liver at various time points post-infection was determined. In the representative experiment reported in Fig. 5, although the counts of the

Fig. 4. Expression of *fadD33* in the *M. tuberculosis* H37Rv-*fadD33*::Kan and *fadD33*-complemented H37Rv-*fadD33*::Kan mutants. The image shows an ethidium-bromide-stained gel of the RT-PCR products that were obtained from RNA extracted from the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant (H37Rv-*fadD33*::Kan), *fadD33*-complemented H37Rv-*fadD33*::Kan (H37Rv-*fadD33*::Kan compl.) and wild-type *M. tuberculosis* H37Rv (H37Rv), and probed for cDNA of *fadD33* and *Rv0441c* (indicated at the bottom). Lanes also include a molecular mass marker (100 bp ladder), PCR carried out in the absence of cDNA (H₂O) and PCR products of DNA from *M. tuberculosis* H37Rv (DNA H37Rv).

inocula yielded the expected numbers of c.f.u.s (approx. 10⁵), the mean number of c.f.u.s of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant found in the spleen at 1 day post-injection was almost 0.4 log₁₀ lower than that of the other strains injected, while it did not differ significantly from the other strains in the lungs and liver. At 14, 42 and 123 days post-infection, the c.f.u. counts of the strains tested were comparable in the lungs and spleen, while the number of c.f.u.s of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant was approximately 0.5 to 0.7 log₁₀ lower than those of the parent strain H37Rv in the liver ($P < 0.02$ at least, by ANOVA). Notably, in the liver, the c.f.u. numbers of the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant complemented with *fadD33* were significantly higher (0.6–1 log₁₀) than those of the *fadD33*-disrupted mutant at 14, 42 and 123 days post-infection ($P < 0.01$, at least) and similar to those of strain H37Rv. No animal deaths were recorded during the whole experimental period. In another preliminary experiment, the survey of the c.f.u. counts in the organs of infected mice was done at 1, 14, 21 and 42 days post-infection, with results comparable to those reported above. Taken together, our findings indicate that disruption of *fadD33* influences the growth of *M. tuberculosis* H37Rv in the liver, but not in the lungs and spleen.

Fig. 5. Experimental infection of BALB/c mice with *M. tuberculosis* H37Rv (■), the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant (▲) and the *M. tuberculosis* H37Rv-*fadD33*::Kan mutant complemented with *fadD33* (●). The data are expressed as the geometric means $\pm$ SD of the log₁₀ c.f.u. counts obtained from four to eight mice per group per time point.

DISCUSSION

In the present study, we have investigated the potential pathogenic role of *fadD33*, a gene of *M. tuberculosis* that is underexpressed in H37Ra, the attenuated strain derived from H37.

As a first step, we tested the possibility that restoration of *fadD33* expression in strain H37Ra might confer growth advantages in a mouse infection model. These experiments showed that complementation of strain H37Ra with *fadD33* restored its replication to the levels of H37Rv in the liver, but not in the lungs and spleen, where the c.f.u. counts were the same for strains H37Ra and H37Rv.

We then used a further experimental approach to test the role of *fadD33* in *M. tuberculosis* virulence. We prepared a *fadD33*-disrupted *M. tuberculosis* H37Rv mutant and the relative *fadD33*-complemented control, and tested them by mouse infection. This approach confirmed the involvement of *fadD33* in the growth of *M. tuberculosis* H37Rv in the liver of BALB/c mice. Indeed, disruption of *fadD33* reduced the growth of *M. tuberculosis* H37Rv in the liver, but not in the lungs and spleen; moreover, complementation of the *fadD33*-disrupted mutant with *fadD33* completely restored bacterial replication in the liver.

An unexpected finding in infection experiments (Fig. 2) was that strains H37Rv and H37Ra replicated to a comparable extent in the lungs and spleen of infected BALB/c mice, although they differed significantly in the liver. Indeed, *M. tuberculosis* H37Rv would be expected to grow rapidly in immunocompetent mice (more than a 2 log₁₀ increase in the c.f.u. count) in the lungs and spleen in the first 2–3 weeks post-infection. The low rate of replication of *M. tuberculosis* H37Rv in our experiments might indicate that the strain we used is not as virulent as others, as also suggested by the results from *in vitro* experiments aimed at investigating the growth of strains H37Rv and H37Ra in human phagocytes. In fact, while *M. tuberculosis* H37Rv was expected to grow significantly faster than H37Ra in human macrophages (McDonough *et al.*, 1993; North & Izzo, 1993; Zhang *et al.*, 1998), in our hands, strains H37Rv and H37Ra showed comparable growth in human peripheral blood monocytes; in activated monocyte-derived THP-1 cells, H37Rv was slightly more resistant than H37Ra to intracellular killing, as judged by the higher number of c.f.u.s detected in cell lysates 2 days post-infection, but grew intracellularly at the same rate as H37Ra thereafter (L. Rindi, unpublished observations). The relatively low rate of replication of our *M. tuberculosis* H37Rv strain in organs of infected mice and in cultured macrophages might be due to the fact that the strain had not been recently passaged *in vivo* nor in macrophages *in vitro*; alternatively, it is also possible that the Rv4 genotype of H37Rv, the variant that we employed in our study (Bifani *et al.*, 2000), is actually poorly virulent for BALB/c mice, a strain considered highly resistant to *M. tuberculosis* (Medina & North, 1998), as well as being only moderately able to grow within macrophages.

Taken together, the experiments employing the *fadD33*-complemented H37Ra and *fadD33*-disrupted H37Rv mutants point to the conclusion that *fadD33* plays a role in *M. tuberculosis* virulence in mice by supporting bacterial growth in the liver. The involvement of mycobacterial *fadD* genes in *M. tuberculosis* tropism is not unprecedented. In fact, it has been reported that the products of genes *fadD26* and *fadD28* participate in the biosynthesis of phthiocerol dimycocerosate, a complex cell-wall-associated lipid found only in pathogenic mycobacteria (Brennan & Nikaido, 1995; Kolattukudy *et al.*, 1997), which determines tissue-specific replication in the lungs (Camacho *et al.*, 1999; Cox *et al.*, 1999). The

reasons why *fadD33* expression might influence *M. tuberculosis* growth in the mouse liver are unknown at present. However, one has to consider that there are 36 *fadD* genes encoding proteins with acyl-CoA synthase activity in *M. tuberculosis* H37Rv and the same number of *fadE* genes encoding putative acyl-CoA dehydrogenases (Cole *et al.*, 1998). It is therefore likely that such a large number of *fadD/fadE* genes in the *M. tuberculosis* genome reflects the necessity of *M. tuberculosis* to load a large variety of lipids onto CoA, some of which have to be transported to specific enzymes for lipid degradation and others of which have to be used for biosynthesis. As *fadD33* is located just upstream of *fadE14* and downstream of an acyl carrier protein gene (*Rv1344*) in the H37Rv genome (Cole *et al.*, 1998), it may be hypothesized that, after intracellular transport, the proteins FadD33 and FadE14 participate in the activation and subsequent oxidation of liver-specific fatty acids needed as a source of carbon and energy by *M. tuberculosis*; the products of these enzymes would be then oxidized further in the two pathways required for fatty acid metabolism in bacteria, i.e. β -oxidation and the glyoxylate shunt (Clark & Cronan, 1996). The importance of fatty acid metabolism in *M. tuberculosis* experimental infection in mice has been underscored by McKinney *et al.* (2000) and Sharma *et al.* (2000), who showed that isocitrate lyase, one of the glyoxylate shunt enzymes, plays a pivotal role for survival of *M. tuberculosis* in the lungs of mice during the persistent phase of infection, entailing a metabolic shift in the carbon source to two-carbon compounds generated by β -oxidation of fatty acids.

Alternatively to a role in the degradation of fatty acids, the enzyme FadD33 might participate, similarly to FadD26 and FadD28 in the lungs (Cox *et al.*, 1999), in the biosynthesis of lipids or other metabolites specifically required for growth of *M. tuberculosis* in the liver of infected mice. Recently, it has been shown that some *fad* and *fadD* genes are induced in *M. tuberculosis* following detergent-induced surface stress (Manganelli *et al.*, 2001), which might suggest a possible role of their products in the biosynthesis of cell-wall lipids. In this context, we also have to consider that FadD33 is similar to the putative polyketide biosynthesis protein PKSJ of *Bacillus subtilis* (Albertini *et al.*, 1995), as it shows a 27.1% identity in an overlap of 468 aa (<http://genolist.pasteur.fr/TubercuList/>). As microbial polyketide metabolites have shown potent biological activities in different models, acting as virulence factors, antibiotics, immunosuppressants and toxins (George *et al.*, 1999; Kolattukudy *et al.*, 1997; Motamedi *et al.*, 1997), it is tempting to consider that mycobacterial FadD33 might be involved in the synthesis of polyketides that play a role in *M. tuberculosis* infection in the liver. The involvement of certain polyketide metabolites in *M. tuberculosis* infection has been reported by Graham & Clark-Curtiss (1999), who showed that *pks2*, one of the 18 polyketide synthase genes of *M. tuberculosis* H37Rv, is expressed in infected human macrophages but not in bacteria growing in culture broth, and by De Voss *et al.*

(2000), who demonstrated that the mycobactin siderophores produced by polyketide synthase enzymes are essential for *M. tuberculosis* growth in macrophages.

Analyses of the lipid profiles of strains H37Rv and H37Ra, as well as the definition of the cellular localization of the *fadD33* gene product, are likely to provide insights into understanding the precise biochemical function of FadD33 and its role in *M. tuberculosis* infection.

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