Selection of transposon mutants of *Mycobacterium tuberculosis* with increased macrophage infectivity identifies *fadD23* to be involved in sulfolipid production and association with macrophages

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Alterations to the composition or architecture of the mycobacterial cell envelope can affect the macrophage infectivity of the bacillus. To further characterize the mycobacterial gene products that modulate the interaction with host cells, we employed transposon mutagenesis and screened for mutants that demonstrated an enhanced binding affinity toward macrophages. After successive rounds of mutant selection and enrichment, a total of five mutants were isolated that harboured gene disruptions within loci involved in lipid synthetic pathways as well as genes coding for putative hypothetical proteins. One mutant in particular, with a disruption in the Rv3826 gene (*fadD23*), was repeatedly isolated during library screening. Analysis of the cell envelope constituents of the Tn::*fadD23* strain revealed a lack of sulfolipid production which was restored following complementation with the wild-type gene.

INTRODUCTION

The World Health Organization estimates that nearly one-third of the global population is infected with *Mycobacterium tuberculosis* (Raviglione, 2003). Whilst the currently available antimicrobials are often effective at eradicating the infection, there are issues with patient drug compliance and the emergence of drug-resistant *M. tuberculosis* (Espinal, 2003; Ormerod, 2005). In addition, the rising incidence of HIV infection, particularly in parts of Africa, has further increased the mortality associated with tuberculosis (Nunn *et al.*, 2005). At the onset of infection in the lung, pathogenic mycobacteria gain access to the alveoli, where the bacteria encounter the resident macrophages. While much is known with regard to macrophage receptors that mediate bacterial recognition and subsequent phagocytosis (El-Etr & Cirillo, 2001; Ernst, 1998), the bacterial surface components that participate in the ligand–receptor interactions remain largely uncharacterized. A thorough understanding of the molecular mechanisms employed by pathogenic mycobacteria when faced with host cells could potentially identify targets for antimicrobial design and lead to the development of novel therapies.

Previously, we reported that disruption of the *M. tuberculosis* surface capsule following sonication significantly increases the level of bacterial association with murine and human macrophages (Stokes *et al.*, 2004). A similar phenotype was also reported following deletion of the *pks3–pks4* locus of *M. tuberculosis*, which reduced the retention of the surface capsule due to a deficiency in mycosanoic and mycolipenic acid synthesis that resulted in the absence of diacyltrehaloses and polyacyltrehaloses in the cell envelope (Dubey *et al.*, 2002; Rousseau *et al.*, 2003). Increased bacterial recognition by macrophages has also been demonstrated in non-tuberculous mycobacteria lacking surface glycopeptidolipids (Etienne *et al.*, 2002). Together these findings suggest that modifications to the mycobacterial cell wall can potentially lead to the exposure of bacterial ligands that are readily recognized by macrophages. Interestingly, a recent study of *M. tuberculosis* clinical isolates reported that strains associated with extra-respiratory sites of infection correlated with an increased level of macrophage infectivity, suggesting that the initial interactions with the host macrophage directly affect bacterial pathogenesis (Garcia de Viedma *et al.*, 2005).

The focus of the present study was to identify and characterize mycobacterial factors that participate in the initial binding interaction with host macrophages. A transposon library of *M. tuberculosis* was screened for mutants that showed an increased level of macrophage

Abbreviation: SL-1, sulfolipid 1.

association as compared to the parental wild-type. Our expectation was that this screening strategy would identify mycobacterial genes that contribute, in some manner, to the biogenesis of the cell wall. A total of five mutants were selected and the sites of transposon disruptions were localized to loci encoding enzymes involved in lipid biosynthesis as well as hypothetical proteins. One mutant containing a transposon disruption within the *fadD23* gene was repeatedly isolated from the library. Lipid analysis of the *fadD23* mutant indicated a deficiency in the production of cell wall sulfolipids. Genetic complementation of the transposon mutant with the wild-type gene led to restoration of the wild-type phenotype for both macrophage binding and sulfolipid production.

METHODS

Bacterial strains. *M. tuberculosis* Erdman (ATCC 35801) was grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5 % glycerol, 10 % oleic acid/albumin/glucose (dextrose) complex (OADC) and 0.05 % Tween or on Middlebrook 7H10 agar (Difco) supplemented with 0.5 % glycerol and 10 % OADC. OADC was prepared as described previously (Dullaghan *et al.*, 2002). For the preparation of lipid extracts, mycobacteria were cultured in glycerol/alanine/salts (GAS) medium (Takayama *et al.*, 1975). *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB-agar plates. When required, hygromycin and kanamycin were used at concentrations of 50 µg ml⁻¹ and 25 µg ml⁻¹ respectively.

Transposon mutagenesis. A transposon library was generated using the pHA87 vector and Tn5370 as described previously (Bardarov *et al.*, 1997). Briefly, a 100 ml culture of *M. tuberculosis* was grown to OD₆₀₀ 0.8–1.0. The cells were washed twice and resuspended in a final volume of 10 ml phage adsorption buffer (10 mM Tris/HCl, pH 7.6, 100 mM NaCl, 10 mM MgSO₄, 2 mM CaCl₂). The bacteria were infected with the phage lysate at an m.o.i. of 10:1 (phage particles:bacteria) for 4 h at 39 °C. The infected cell suspension was centrifuged at 16 000 g for 5 min to recover the bacteria before plating onto selective media. After 4–6 weeks of growth, hygromycin-resistant colonies were scraped from the agar plates and cultured in 7H9 in separate pools each containing roughly 500 mutants. After 7–10 days of growth, the cultures were aliquoted and stored at –80 °C. A total of 20 library pools were used in the screening experiments.

Southern blot analysis on a total of 38 randomly selected mutants confirmed that significant diversity in mutant genotypes was present in the transposon library (data not shown). Briefly, genomic DNA from the 38 mutants was digested with *PvuII*, which does not cut within the transposon, and transferred onto nylon membrane as described elsewhere (Sambrook *et al.*, 1989). The blots were hybridized with a DIG-labelled DNA fragment derived from the hygromycin-resistance cassette according to the directions of the manufacturer (Roche Applied Science). The insertion patterns and limitations of Tn5370 transposon have been previously characterized (McAdam *et al.*, 2002). Thus, while the library may not be representative of the whole genome, it is likely that insertions are present in a significant number of the genes.

Infection of THP-1 cells. The human macrophage-like cell line THP-1 (ATCC TIB202) was cultured in RPMI 1640 (Gibco) supplemented with 10 % heat-inactivated fetal calf serum (Gibco), 10 mM L-glutamine (Gibco) and 10 mM sodium pyruvate (Gibco) and maintained in an atmosphere of 5 % CO₂ at 37 °C as described

previously (Stokes & Doxsee, 1999). For infection assays, THP-1 cells were seeded onto acid-washed glass coverslips (2 × 10⁵ cells per coverslip) or into T-75 cm² flasks (5 × 10⁶ cells) and differentiated in the presence of 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) over 3 days. Prior to infection, the monolayers were washed with binding medium to remove any residual PMA.

Single-cell suspensions of mycobacteria for use during infection assays were prepared using two separate techniques, essentially as described previously (Stokes *et al.*, 2004). Briefly, the bacteria were pelleted by centrifugation (16 000 g for 5 min) and resuspended in 500 µl binding medium (138 mM NaCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.7 mM KCl, 0.6 mM CaCl₂, 1 mM MgCl₂ and 5.5 mM D-glucose; Smith & Iden, 1981). The bacterial clumps were then dispersed by 10 repeated passages through a 25-gauge needle attached to a 1 ml syringe or by three 30 s pulses of bath sonication using a VC50T 50 W microcup horn (Sonics & Materials) as indicated for each experiment (Stokes *et al.*, 2004). The volume of the cell suspension was then adjusted using binding medium to achieve the desired bacterial concentration.

The differentiated THP-1 cells were infected with mycobacteria for two purposes: (i) to screen the transposon library and (ii) to test the macrophage binding affinities for the mutant clones selected for further study. For procedure (i), THP-1 cells cultured in T-75 cm² flasks and on coverslips were infected with the library pools (synting suspensions) at an m.o.i. of 100:1 (bacteria:macrophages) for 4 h. Non-adherent mycobacteria were removed by vigorously washing the monolayer three times with binding medium (10 ml for flasks, 500 µl for coverslips), a method that we have previously shown to be effective for the removal of non-adherent bacteria (Stokes *et al.*, 1986). The infected coverslips were fixed (10 min in 10 % formaldehyde in ethanol) and mounted onto microscope slides followed by staining with Kinyoun's Carbol Fuchsin and malachite green. The level of bacterial binding for each mutant pool was determined by microscopic count. The infected monolayers in the T-75 cm² flasks were used to enrich for mutants with enhanced host-cell-binding capabilities; the THP-1 cells were dislodged using a cell scraper, resuspended in PBS containing 0.1 % Tween-80 (PBS-Tween) and briefly sonicated to lyse the macrophages. The macrophage-associated bacteria were recovered and cultured for 7–10 days in 7H9 before being used as the inoculum in a subsequent round of selection. After six to eight rounds of enrichment, the library pools that displayed an increased level of macrophage association (as determined by microscopic count) were plated onto selective agar plates and individual mutant clones were assessed for macrophage infectivity.

For procedure (ii), syringed and sonicated suspensions of mycobacteria were prepared as described above and used to infect THP-1 monolayers on coverslips at an m.o.i. of 100:1 (bacteria:macrophages) for 3 h. The monolayers were fixed and stained as described above. The percentage of infected macrophages was determined by counting 100 macrophages for each coverslip.

Identification of the transposon insertion site. Genomic DNA from the selected transposon mutants was extracted (Belisle & Sonnenberg, 1998), restriction digested with *PvuII* and analysed by Southern hybridization using a labelled fragment from the hygromycin-resistance cassette. The presence of a single hybridizing DNA fragment on the Southern blot verified that a single copy of the transposon had incorporated into the chromosome. The precise location of the transposon insertion was determined by sequencing the amplicons generated by ligation-mediated inverse PCR (Cox *et al.*, 1999). Briefly, 2.5 µg DNA was digested with *RsaI*. The restriction digest was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. An aliquot of the digested DNA (~200 ng) was ligated overnight at 16 °C, followed by ethanol

precipitation. The DNA sequences adjacent to the transposon were amplified using primers corresponding to the left (O84L-F 5'-GTCATCCGGCTCATCACCAG-3' and O84L-R 5'-AACTGGC-GCAGTTCTCTGG-3') and right (O84R-F 5'-ATACACGCGCAC-CGGTTCTAGC-3 and O84R-R 5'-CACGGCGAACCCTGGTG-3') ends of the transposon. Sequence comparisons were done using BLAST 2.0 at www.ncbi.nlm.nih.gov/. The transposon insertion within the *fadD23* gene was also confirmed by PCR using primers that flank the site of transposon integration; the FADD-L 5'-TCTGA-CAACATCCGCGATAA-3' primer corresponds to nucleotides 348–368 and the FADD-R 5'-GTCACCTCGAAGGCGAAGTT-3' primer matches nucleotides 807–827 of the *fadD23* gene.

Complementation of the *fadD23* transposon mutant. The complementation construct was generated by amplifying the *fadD23* gene along with 455 bp of upstream sequence from *M. tuberculosis* chromosomal DNA using *Pfu* DNA polymerase (Stratagene) with the following primers (FADJLL 5'-CGTAGAACTCGTCGAATCC-3' and FADJLR 5'-GTGGATCCGACCGTAAGACC-3'). The amplicon was blunt-end ligated into the mycobacterial integration vector pMV306, which had been digested with *EcoRV* (Stover *et al.*, 1991). The resulting plasmid construct was sequenced and transformed into the mutant by electroporation as described elsewhere (Parish & Stoker, 1998). Transformed bacteria were recovered using kanamycin selection and the presence of the wild-type *fadD23* gene in the complemented mutant was confirmed by PCR using the FADD-L and FADD-R primers.

Lipid analysis. Apolar and polar lipids were extracted from bacterial cultures that were pelleted by centrifugation, autoclaved (121 °C, 60 min) and subsequently lyophilized as described previously (Besra, 1998). Lipids were separated by two-dimensional thin-layer chromatography (2D-TLC) on Silica Gel 60 plates (EM Science) using five different solvent systems (A–E) for mycobacterial lipid analysis that have been described elsewhere (Besra, 1998; Dobson *et al.*, 1985). Sulfolipids were localized using solvent system D, which uses chloroform/methanol/water (100:14:0.8) for the first-dimension solvent system and chloroform/acetone/methanol/water (50:60:2.5:3) for the second dimension. Lipids were visualized by spraying TLC plates with either 5 % ethanolic phosphomolybdic acid or 2.5 % α -naphthol/10 % sulfuric acid followed by charring. Sulfolipid 1 (SL-1) was also detected by staining TLC plates with cresyl violet as described elsewhere (Soto *et al.*, 2000). Briefly, the lipids were resolved on TLC plates using solvent system D and the plates were immersed in a solution of 0.02 % cresyl violet in 1 % aqueous acetic acid for 10 min followed by briefly destaining with 1 % aqueous acetic acid (data not shown).

Statistical analysis. The results shown are the mean $\pm$ SEM. Statistically significant differences between groups were determined with the two-tailed, unpaired Student's *t* test or analysis of variance. *P* values <0.05 were considered significant.

RESULTS

Transposon mutagenesis and mutant selection

A transposon library using *M. tuberculosis* Erdman was generated using the Tn5370 transposon in conjunction with a temperature-sensitive mycobacteriophage delivery vector (Bardarov *et al.*, 1997). A biological screening assay consisting of iterative rounds of macrophage infection was used to select for transposon mutants with an enhanced ability to bind macrophages. As outlined in Fig. 1, 20 pools

from a transposon library (each containing ~500 mutants) of *M. tuberculosis* were used to infect THP-1 cell monolayers. Transposon mutants that preferentially associated with host cells were recovered from the monolayer and used as the initial inoculum in a subsequent macrophage infection assay. We hypothesized that the desired phenotype would become enriched within the output mutant population after successive rounds of selection. Between the sixth and eighth round of successive macrophage infection assays, three of the library pools emerged that consistently bound the cell monolayers more readily than the syringed *M. tuberculosis* control (Fig. 2). The three pools were plated onto 7H10 agar and 40 mutants from each pool were isolated at random. Southern blotting was used to identify siblings within each pool (data not shown) and the level of macrophage association was determined for each of the 40 mutants from the three pools (a total of 120 mutants). The site of transposon insertion was identified using inverse PCR and DNA sequencing of mutants within the pools with the highest levels of macrophage association (Table 1). None of the mutants appeared to have any alterations in colony morphology or growth rate in broth when compared to the wild-type (data not shown).

Fig. 1. Library screening to identify mutants with an increased macrophage binding affinity. (A) A transposon library was generated using the Tn5370 transposon. (B) The library was subdivided into 20 pools each containing 500 mutants. (C) The mutant pools were serially passaged using binding to THP-1 cells as the selection criterion. (D) Cell-associated bacteria were recovered from the monolayer. (E) These bacteria were cultured in broth and used as the infecting inoculum in the next round of selection. (F) Three mutant pools that showed increased levels of macrophage binding were plated onto 7H10 agar. (G) Mutants from each of the selected pools were tested for their ability to bind macrophages and Southern hybridization was used to identify siblings. The site of transposon insertion was determined by inverse PCR.

Fig. 2. Pools of mutants that exhibit increasing levels of macrophage association. During library screening, three mutant pools (A–C) were identified that showed increasing levels of macrophage association with each round of selection. The level of macrophage binding was determined by microscopic count of fixed monolayers. The graphs represent the percentage of macrophages binding >10 syringe-dispersed (see Methods) *M. tuberculosis* (white bars) or >10 syringe-dispersed bacteria from the library pool (black bars). The data shown are the means $\pm$ SEM of three infected coverslips. * denotes $P < 0.05$ when compared to syringe-dispersed wild-type *M. tuberculosis*.

As shown in Fig. 3, all of the mutants identified from the selection procedure exhibited a binding phenotype that more closely mimicked that of the sonicated preparation of wild-type *M. tuberculosis*. These increases in macrophage infectivity suggested that the transposon insertions led to an altered bacterial surface and increased recognition by the host macrophages. To confirm that the increases in bacterial binding were not merely a side effect of the mutagenesis procedure, we included a transposon mutant (mutant 1) from library pool A as a negative control. As shown in Fig. 3, the mutant 1 strain did not exhibit any increase in macrophage association and was indistinguishable from a syringed preparation of wild-type *M. tuberculosis*, suggesting that the phenotypes of the mutants

identified from the screening process could not be simply attributed to the transposon construct. It is also important to note that the transposon mutants that were identified from the screening procedure did not exhibit any increased bacterial clumping when cultured *in vitro* and that the syringe-dispersal treatment used prior to macrophage infection was sufficient for the disruption of small clumps of mycobacteria, as previously described (Stokes *et al.*, 2004)

Lipid analysis of the *fadD23::Tn5370* mutant

Among the five transposon mutants that were identified from the library screen, the *fadD23* transposon mutant was repeatedly isolated from two of the three library pools that demonstrated an enhanced macrophage-binding phenotype: the transposon disruption in *fadD23* was detected in five of the 40 mutants from pool A tested by Southern analysis and two of 40 mutants from pool B. The transposon insertion mapped to nucleotide 630 of the *fadD23* gene, shown in Fig. 4(a). The *fadD23* mutant displayed an increased binding affinity to THP-1 cells as compared to wild-type ($P < 0.05$, ANOVA). Complementation of the mutation with the *fadD23* gene *in trans* resulted in binding levels that were intermediate between the wild-type and the mutant, and not significantly different from either ($P > 0.05$, ANOVA), indicating that partial restoration of the binding phenotype was achieved following complementation (Fig. 4b). The close proximity of the *fadD23* gene to other loci that have been linked to sulfolipid biosynthesis prompted us to investigate whether the biosynthesis of cell wall glycolipids was affected in the *fadD23* transposon mutant. The apolar and polar lipid fractions were extracted from whole-cell pellets of the parental wild-type, the mutant and the complemented strain for analysis by TLC. As shown in Fig. 5, comparison of the apolar lipid fractions revealed a lack of sulfolipid production in the absence of *fadD23*. Confirmation that the lipid fraction was indeed sulfolipid was obtained by staining the TLC plates with cresyl violet (Soto *et al.*, 2000) (data not shown). Importantly, sulfolipid production could be restored following complementation of the mutant with the wild-type *fadD23* gene.

DISCUSSION

To date much of the research surrounding the interaction between *M. tuberculosis* and host macrophages has centred on the host-cell receptors that mediate bacterial internalization. Furthermore, while great strides have been made in elucidating the chemical composition of the mycobacterial cell envelope, much less is known about the biological function of the different cell wall moieties during infection. Previous findings from our group have indicated that certain mechanical treatments that are used to generate single-cell suspensions of mycobacteria affect both the

Table 1. Mutants isolated from the transposon library that exhibit an increased binding affinity to THP-1 cells

<i>M. tuberculosis</i> H37Rv gene ID	Gene name	Site of insertion/location on genome (nucleotides)*	Predicted function
Rv3826	<i>fadD23</i>	+ 630/4 300 442	Fatty-acyl AMP ligase
Rv1505c	<i>Rv1505c</i>	+ 335/1 695 611	Conserved hypothetical protein
Rv0405	<i>pks6</i>	+ 1237/486 968	Polyketide synthase
Rv3335c	<i>Rv3335c</i>	+ 663/3 721 937	Probable conserved integral membrane protein
Rv1115	<i>Rv1115</i>	−28/1 240 159	Possible exported protein

*The site of insertion is relative to the coding sequence of the gene as determined using Tuberculist (<http://genolist.pasteur.fr/TubercuList/>). The location on the genome is relative to the initiation codon of the *dnaA* gene as described previously (Cole *et al.*, 1998).

bacillus surface and the level of interaction with host macrophages (Stokes *et al.*, 2004). We hypothesized that this observation could be used as a library screening tool to identify mutants that had incurred cell wall alterations as a result of mutagenesis. In this investigation, we screened a transposon library of *M. tuberculosis* using successive rounds of infection assays with THP-1 cell monolayers. Five mutants were identified from the screen that exhibited an increased binding affinity for host cells as compared to the parental wild-type, and at least three of these disrupted

genes (*Rv3335c*, *pks6* and *Rv1115*) have been previously linked to mycobacterial pathogenesis.

The *pks6* gene encodes a predicted polyketide synthase that was initially identified by signature-tagged mutagenesis and mutants in this gene were found to be impaired for growth in the lungs of Balb/c mice (Camacho *et al.*, 1999). Separate studies have also suggested that Pks6 is involved in countering host defences that involve interferon- γ (Hisert *et al.*, 2004). Although the precise biological function of Pks6 is unknown, a recent report suggests its involvement in the synthesis of polar lipids within the *M. tuberculosis* cell envelope (Waddell *et al.*, 2005). The *Rv1115* and *Rv3335c* genes encode hypothetical proteins for which there is little functional information, although these loci have been previously identified from separate transposon libraries; a transposon mutant in *Rv3335c* has been previously shown to be defective for growth within the spleens of C57/BL6 mice using the transposon-site hybridization (TRASH) system (Sasseti *et al.*, 2003) and disruption of the *Rv1115* gene in *Mycobacterium bovis* BCG leads to increased accumulation of the mutant within acidified phagosomes following macrophage infection (Stewart *et al.*, 2005). The *Rv1505c* gene encodes a predicted protein with a conserved domain that is associated with enzymes belonging to the hexapeptide repeat superfamily of acetyltransferases (Raetz & Roderick, 1995; Vuorio *et al.*, 1994; Wenzel *et al.*, 2005). It is important to note that confirmation of the phenotypes for the *Rv3335c*, *pks6*, *Rv1115* and *Rv1505c* mutants will require genetic complementation to rule out the possibility of polar effects. In particular, the ORFs downstream from the *Rv1505c* and *Rv1115* genes may be affected as a result of the transposon insertions. The *Rv1505c* gene appears to reside within an operon that encompasses *Rv1505c*–*Rv1503c*. The *Rv1503c* and *Rv1504c* genes, which are immediately downstream of *Rv1505c*, belong to the DegT/DnrJ/EryC1/StrS aminotransferase family in the protein families (Pfam) database (Bateman *et al.*, 2004). Interestingly, the Rv1503–Rv1504 proteins share a high degree of similarity with the WecE protein (59 % identity, 71 % similarity) of *E. coli* and *Salmonella enterica*. Within

Fig. 3. Macrophage binding of mutants identified by library selection. The individual mutant clones (*fadD23*::Tn5370, *Rv1505c*::Tn5370, *pks6*::Tn5370, *Rv3335c*::Tn5370 and *Rv1115*::Tn5370) were cultured to exponential phase and syringe-dispersed (see Methods) prior to infecting THP-1 macrophages. Wild-type *M. tuberculosis*, either syringe-dispersed (MTB SYR) or sonicated (MTB SON), were included as controls. A transposon mutant from the library (mutant 1) was also included as a control for non-specific effects of the transposon. Black bars represent the percentage of macrophages associated with at least one bacterium; hatched bars represent the percentage of macrophages associated with >10 bacteria. The mean $\pm$ SEM is shown for two independent experiments each with triplicate coverslips. * $P < 0.05$, ** $P < 0.001$ when compared to syringe-dispersed wild-type *M. tuberculosis*.

Fig. 4. Disruption of the *fadD23* gene results in increased association between *M. tuberculosis* and macrophages. (a) Diagram of the genomic region that encompasses the *fadD23* gene, showing the site of transposon mutagenesis. (b) Association of wild-type *M. tuberculosis* (MTB), *fadD23::Tn5370* and the complemented strain (Comp.) with THP-1 monolayers. The mean $\pm$ SEM is shown for three independent experiments, each with triplicate coverslips. * $P < 0.05$ when comparing the *fadD23* mutant to wild-type *M. tuberculosis*.

these enteric pathogens, WecE is a sugar aminotransferase that functions in the synthesis of the carbohydrate component of the enterobacterial common antigen, a glycolipid located on the outer leaflet of the Gram-negative outer membrane. In the case of the *Rv1115* transposon mutant, the downstream locus (*Rv1116*) is located just over 100 bp downstream and encodes a hypothetical protein of unknown function. Polar effects from the transposon are less likely to be an issue with the *pks6* and *Rv3335c* mutants since the downstream loci are not cotranscribed in the same orientation.

The *fadD23* transposon mutant was chosen for further study as this mutation was repeatedly identified within the output population of mutants that remained after the selection assays. The *fadD23* gene is located upstream of two loci that have been confirmed to be involved in sulfolipid biosynthesis. The *Pks2* polyketide synthase catalyses the formation of the sulfolipid acyl chains (Sirakova *et al.*, 2001) while the *MmpL8* protein mediates the transport of mature sulfolipid or its precursors from the cytosol into the cell envelope (Converse *et al.*, 2003; Domenech *et al.*, 2004). The *fadD23* gene probably encodes

a fatty-acyl CoA ligase that would function in concert with the *Pks2* enzyme to generate the glycolipid acyl chains (Trivedi *et al.*, 2004). The *fadD23* gene is flanked on the other side by the *Rv3827c* gene, which encodes a putative transposase. It is unlikely that polar effects play a role in the phenotype of the *fadD23* mutant given that the transcription of the *Rv3827c* gene is in the opposite orientation relative to *fadD23* and that mutant complementation was sufficient to restore the wild-type phenotype. Thus from the data presented in this report, we propose that the *FadD23* protein should now also be included in the list of SL-1 biosynthetic enzymes. Comparative TLC analysis of the apolar lipid fractions from the parental wild-type and the *fadD23* transposon mutant confirmed the absence of mature SL-1 on the cell surface of the mutant strain. In addition, SL-1 production could be restored by complementation of the mutant with the *fadD23* gene. We found that disruption of the *fadD23* gene resulted in an increased level of bacterial association with macrophages which was restored to wild-type levels following complementation.

The correlation of SL-1 loss with increased binding of *M. tuberculosis* to macrophages could reflect alterations in the

Fig. 5. Disruption of *fadD23* in MTB leads to a loss of SL-1 production. Apolar lipids from *M. tuberculosis* (MTB), *fadD23::Tn5370* and the complemented strain (Complement) were separated by 2D-TLC to resolve SL-1. Plates were stained with α -naphthol and charred. DAT, diacyltrehalose; CF, cord factor; SL-1, sulfolipid-1; ?, unknown lipid.

organization of the cell envelope leading to the exposure of previously masked bacterial ligands. Alternatively, the block in sulfolipid production seen in the mutant may lead to an upregulation in the synthesis of other cell wall lipids. Recently, Jain *et al.* (2007) reported that PDIM mutants of *M. tuberculosis* synthesized an increased abundance of sulfolipid due to the increased availability of methylmalonyl-CoA precursors. While the authors did not identify a reciprocal increase in PDIM synthesis in SL-1 deficient mutants (Jain *et al.*, 2007), there remains the possibility that the transposon mutation in *fadD23* may cause a disturbance in the total distribution of cell wall lipids. Finally, the absence of SL-1 may also affect the overall surface charge of the bacillus, thereby increasing the level of cell–cell interactions. In support of this possibility, we have previously shown a correlation between a negatively charged mycobacterial surface and enhanced macrophage association (Stokes *et al.*, 2004).

There are other examples in the literature where loss of cell surface lipids has resulted in improved association of mycobacteria and host cells. *Mycobacterium smegmatis* mutants lacking cell wall glycopeptidolipids (GPL) are more rapidly internalized by macrophages than are wild-type bacteria (Etienne *et al.*, 2002), perhaps due to the inhibitory effects of GPL on *M. smegmatis* binding (Villeneuve *et al.*, 2003). In addition, inactivation of the *pkc3–pkc4* locus in *M. tuberculosis* coincides with an increase in macrophage binding due to loss of diacyltrehaloses and polyacyltrehaloses (Rousseau *et al.*, 2003). Collectively, these and our earlier findings (Stokes *et al.*, 2004) suggest that the interaction of mycobacteria with macrophages is limited by the presence of an intact outer cell envelope, disruption of which leads to enhanced binding.

In summary, transposon mutants were identified that were more apt to associate with phagocytic cells, implying that these strains had sustained some cell surface alterations responsible for their increased uptake. Lipid analysis of the *fadD23* transposon mutant confirmed the involvement of *fadD23* in the synthesis of cell wall sulfolipid. However, it is important to note that the collection of mutants identified in this study does not represent an exhaustive list of all the mycobacterial genes involved in binding to host macrophages. In particular, the use of large pools of mutants as part of the screening process does not favour the selection of mutant clones that have reduced rates of *in vitro* replication. There is also the possibility that neighbouring bacteria may complement the phenotypes of other mutants within the same pool (Sasseti & Rubin, 2003). Further investigation will focus on the cell wall compositions of the remaining mutants and will determine whether the alterations in the cell envelope correlate with changes in bacterial virulence.

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