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β-lactamase can function as a reporter of bacterial protein export during *Mycobacterium tuberculosis* infection of host cells

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Summary

Mycobacterium tuberculosis is an intracellular pathogen that is able to avoid destruction by host immune defenses. Exported proteins of *M. tuberculosis*, which include proteins localized to the bacterial surface or secreted into the extracellular environment, are ideally situated to interact with host factors. As a result, these proteins are attractive candidates for virulence factors, drug targets, and vaccine components. Here we describe a new β-lactamase reporter system capable of identifying exported proteins of *M. tuberculosis* during growth in host cells. Because β-lactams target bacterial cell wall synthesis, β-lactamases must be exported beyond the cytoplasm to protect against these drugs. When used in protein fusions, β-lactamase can report on the subcellular location of another protein as measured by protection from β-lactam antibiotics. Here we demonstrate that a truncated TEM-1 β-lactamase lacking a signal sequence for export (‘BlaTEM-1) can be used in this manner directly in a mutant strain of *M. tuberculosis* lacking the major β-lactamase, BlaC. The ‘BlaTEM-1 reporter conferred β-lactam resistance when fused to both Sec and Tat export signal sequences. We further demonstrate that β-lactamase fusion proteins report on protein export while *M. tuberculosis* is growing in THP-1 macrophage-like cells. This genetic system should facilitate the study of proteins exclusively exported in the host environment by intracellular *M. tuberculosis*.

INTRODUCTION

Tuberculosis is responsible for nearly two million deaths each year (World Health Organization, 2007). *Mycobacterium tuberculosis*, the causative agent of this disease, is an intracellular pathogen and the ability of this bacterium to survive and grow in macrophages is essential to its virulence. Multiple processes are likely employed by *M. tuberculosis* to avoid destruction in macrophages. These include residing in a phagosome that fails to mature into an acidified phagolysosome and resisting reactive radicals (as reviewed in Russell, 2007 and Zahrt & Deretic, 2002). As in other bacterial pathogens, *M. tuberculosis* proteins exported beyond the cytoplasm to the bacterial cell envelope (comprised of the cytoplasmic membrane and cell wall) or secreted into the environment are ideally positioned to interact with host cell components and promote survival in macrophages. Consequently, exported and secreted proteins make good candidates for virulence factors, drug targets for disease intervention, and vaccine antigens.

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Mycobacteria possess two conserved pathways for exporting proteins: the general secretion (Sec) pathway and the twin-arginine translocation (Tat) pathway (Braunstein *et al*., 2001; Kurtz & Braunstein, 2005; McDonough *et al*., 2005; Owens *et al*., 2002; Posey *et al*., 2006; Saint-Joanis *et al*., 2006). These systems recognize precursor proteins synthesized with amino-terminal signal sequences and transport them across the cytoplasmic membrane (DeLisa *et al*., 2003; Mori & Ito, 2001). The proteins exported by these pathways can remain associated with the cell envelope or be further secreted by the bacterium. The signal sequences of Sec and Tat substrates share a similar domain structure; however, Tat substrates are distinguished by the presence of the twin-arginine motif, R-R-x-ϕ-ϕ (ϕ = uncharged residue). The two pathways also differ in their mode of transport. Sec substrates are translocated across the cytoplasmic membrane in an unfolded state, whereas Tat substrates are translocated in a folded conformation. *M. tuberculosis* also has at least two specialized protein export pathways: the SecA2-dependent system and the ESX-1 (ESAT-6) system (Braunstein *et al*., 2003; Guinn *et al*., 2004; Hsu *et al*., 2003; Pym *et al*., 2003; Stanley *et al*., 2003). Interestingly, both pathways appear capable of secreting specific subsets of proteins that lack conventional Sec or Tat signal sequences.

In *M. tuberculosis*, proteomic and genetic methods have been used to experimentally identify proteins exported beyond the cytoplasm (reviewed in Kurtz & Braunstein, 2005). The genetic methods rely on reporter enzymes that are fused to *M. tuberculosis* protein sequences and report on the subcellular location of the fusion proteins (Braunstein *et al*., 2000; Chubb *et al*., 1998; Downing *et al*., 1999; Lim *et al*., 1995; Wiker *et al*., 2000). Surrogate hosts such as non-pathogenic *Mycobacterium smegmatis* or *Escherichia coli* have been used in most of these studies, often because endogenous enzyme activities in *M. tuberculosis* precluded their use directly in the pathogen. The use of surrogate hosts is a problem for identifying proteins that are only exported by pathogenic *M. tuberculosis*.

β-lactamase is an export reporter that was not initially employed directly in *M. tuberculosis* because of endogenous β-lactam resistance. β-lactamase catalyzes the hydrolysis of β-lactams, a class of antibiotic that targets cell wall biosynthetic enzymes located outside of the cytoplasmic membrane. Therefore, β-lactamase must be exported beyond the cytoplasm to protect the bacterium from the drug. For this reason, when fused to another protein, it can be used as an export reporter with β-lactam resistance as a powerful indicator of export. We recently reported that a *ΔblaC* mutant of *M. tuberculosis*, lacking the chromosomally encoded β-lactamase BlaC, is β-lactam sensitive (Flores *et al*., 2005). Further, we showed that BlaC is a native Tat substrate and that a truncated ‘BlaC lacking a signal sequence can function as a reporter of Tat-dependent export directly in a *ΔblaC* mutant of *M. tuberculosis* (McDonough *et al*., 2005). This was shown by fusing a Tat signal sequence to ‘BlaC and demonstrating that the resulting hybrid protein confers resistance to the β-lactam antibiotic carbenicillin in the *ΔblaC* background. Interestingly, the ‘BlaC reporter works with Tat but not Sec exported proteins. Here we expanded the β-lactamase tools that can be used directly in *M. tuberculosis* by demonstrating that the TEM-1 β-lactamase (BlaTEM-1), originally identified in a clinical isolate of *E. coli* (Datta & Kontomichalou, 1965), functions as an export reporter in the *ΔblaC* mutant of *M. tuberculosis*. The ‘BlaTEM-1 reporter has the significant advantage of being compatible with both Sec and Tat signal sequences.

The proteomic and genetic approaches used in previous work for identifying exported proteins of *M. tuberculosis* are limited by their reliance on *in vitro* grown bacteria. Consequently, a potentially interesting collection of proteins only exported or secreted while *M. tuberculosis* are inside host cells are missed. In this report, we demonstrate that β-lactamase reporters have the novel capability of identifying *M. tuberculosis* proteins that are exported during intracellular growth in β-lactam treated THP-1 macrophage-like cells. The system we describe will be of significant value for identifying the most interesting category of exported *M. tuberculosis* proteins – those exported during growth in the host environment.

METHODS

Bacterial strains, media and growth conditions

Escherichia coli DH5α was grown in Luria-Bertani medium (Fisher) supplemented with the following concentrations of antibiotics as required: carbenicillin, 100 μg/ml; kanamycin, 40 μg/ml. *M. tuberculosis* strains H37Rv (WT), PM638 (*ΔblaC*, H37Rv) (Flores *et al*., 2005) and all derivative strains were cultured in Middlebrook 7H9 medium or on Middlebrook 7H10 agar medium (Difco; BD Biosciences) supplemented with 10% ADS (0.5% BSA, fraction V [Roche]; 0.2% dextrose; and 0.85% NaCl), 0.5% glycerol, and 0.05% tween 80 (Fisher). Antibiotics for mycobacteria were used at the following concentrations: carbenicillin, 50 μg/ml; kanamycin, 20 μg/ml. 7H10 plates supplemented with carbenicillin lacked tween, as the combination of tween and carbenicillin appeared detrimental to growth of fusion-expressing strains.

Construction of ‘*blaTEM-1* fusion plasmids

Plasmids used in this study are listed in Table 1. All subcloned PCR products were sequenced and determined to be error free. Sequence encoding the mature domain (lacking the N-terminal signal sequence) of *E. coli* BlaTEM-1 was amplified from pUC19 plasmid DNA (Invitrogen) using the following primers: TEMbla1 (5’-AGATCTCACCCAGAAACGCTGGTGAAAG) and TEMbla2 (5’-GTTACCAATGCTTAATCAGTGAGGCACC). The resulting PCR product was cloned into the pCC1 vector (Epicentre) to generate pJM114. The ‘*blaTEM-1* reporter was subcloned as a *Bgl*II-*Bam*HI fragment into each of the multi-copy vectors described below. **(i) Δss, ‘*blaTEM-1***. ‘*blaTEM*-1 was digested from pJM114, end-filled with Klenow and cloned into *Msc*I cut pMV261. The resulting plasmid, pJES102, contains the ‘*blaTEM-1* reporter without a fused signal sequence cloned downstream of the *hsp60* promoter. **(ii) ss*plcB*-‘*blaTEM-1***. The ‘*blaTEM-1* fragment was subcloned into *Bam*HI cut pMB222. The resulting plasmid, pJES101, contains an in-frame fusion of DNA encoding the signal sequence of PlcB/Rv2350c (*ssplcB*) to ‘*blaTEM-1* under the control of the *hsp60* promoter. **(iii) ss*mpt63*-‘*blaTEM-1***. The ‘*blaTEM-1* fragment was subcloned into *Bam*HI cut pMB227. The resulting plasmid, pJES103, contains an in-frame fusion of ss*mpt63* (*Rv1926c*) to ‘*blaTEM-1* under the control of the *hsp60* promoter. **(iv) ss*mpt83*-‘*blaTEM-1***. DNA encoding the signal sequence and the first 31 amino acids of the mature *M. tuberculosis* Mpt83 (Rv2873) protein along with the native *mpt83* promoter (Juarez *et al*., 2001) was amplified from *M. tuberculosis* genomic DNA using the following primers: mpt83HindIIIF (5’-CAAGCTTCGTCGGATCCGTGGTAGGGGATGTC) and mpt83HindIIIR (5’-CAAGCTTCGGGGTCAGCCATTGCCGCCGTGG) and cloned into the pCR2.1 vector (Invitrogen) to generate pJES125. A *Hin*dIII fragment from pJES125, carrying ss*mpt83* and upstream genomic sequence, was cloned into *Hin*dIII cut pJES128 (Table 1). The resulting plasmid, pJES129, contains an in-frame fusion of ss*mpt83* to ‘*blaTEM-1* under the control of the native *mpt83* promoter (P_{mpt83}).

Protein quantification by immunoblot

Whole cell lysates of *M. tuberculosis* strains were prepared as described previously (Braunstein *et al*., 2001) with the following modifications. *M. tuberculosis* cultures were grown in 5 ml volumes to mid-exponential phase. The cultures were washed twice and resuspended in PBS 0.02% Tween 80. An equal volume of 10% formalin was added to the washed cultures, which were then incubated at room temperature for 1 hour with frequent mixing by inversion. The formalin fixation step was necessary to kill *M. tuberculosis* before further processing. Bacteria were then harvested by centrifugation at 3000 RPM, washed once in PBS 0.02% tween to remove residual formalin, and bead-beaten lysates were then obtained from each sample. Protein concentration for each lysate was measured using a bicinchoninic acid protein quantification kit (Pierce). Lysates were boiled for 10 minutes, subjected to SDS-PAGE and immunoblots were performed using standard conditions. Primary antibodies specific for BlaTEM-1 were used at a concentration of 1:5000 (QED Biosciences), and horseradish peroxidase-conjugated anti- mouse secondary antibodies were used at a concentration of 1:20,000. Bands were visualized using Western Lightning Chemiluminescent Reagent Plus (PerkinElmer) and quantified using ImageJ Image Processing and Analysis software (http://rsb.info.nih.gov/ij/). Whole cells lysates with the highest level of expression were diluted to enable direct comparison of all hybridization signals on a single blot. The comparative quantification was determined by measuring pixel density of an equal area for each blotted lysate in duplicate. Signal intensity per µg of whole cell lysate loaded was determined and is reported as the amount relative to protein detected in the ‘BlaTEM-1 expressing strain.

Macrophage infections

THP-1 cells were maintained in RPMI (Gibco)/10% heat inactivated fetal calf serum (FCS) at 37 °C and 5% CO_2. To prepare THP-1 monolayers for infection, cells were spun down at 300 *g*, washed once in RPMI, then resuspended in RPMI/10% FCS at a concentration of 1×10^6 cells/ml. Cells were seeded into 8-well tissue culture slides at 2×10^5 cells/well and treated with phorbol myristate acetate (PMA) at a final concentration of 50 ng/ml for 48 hours.

M. tuberculosis was grown to mid-exponential phase OD_{600} of 0.5–1.0. Immediately prior to infection, the bacterial culture was pelleted, washed once in PBS containing 0.05% Tween 80 (PBS-Tw), and resuspended in an equal volume of PBS-Tw. The culture was then briefly sonicated to break up clumps of bacteria, diluted in RPMI/10%FCS medium and added to the THP-1 monolayer at m.o.i. = 0.1.

THP-1 monolayers were infected with *M. tuberculosis* strains for 4 hours at 37 °C and 5% CO_2. Overlaying medium was then removed, the monolayers were washed 3 times with RPMI to remove non-cell associated bacteria, and triplicate wells were lysed and plated to determine uptake (day 0 time-point). The infected monolayers were then overlaid with RPMI/10% FCS, or RPMI/10% FCS supplemented with carbenicillin and maintained at 37 °C and 5% CO_2. At 3 days post infection, the overlying medium was replenished with RPMI/10% FCS media or media supplemented with carbenicillin, as appropriate. On days 1, 3 and 5 post-infection, triplicate wells for each infection were washed to remove antibiotic and lysed with 0.05% SDS. The resulting lysates were diluted and plated on 7H10 agar to enumerate intracellular bacteria during the course of infection. On day 0 and day 5 of the infection, cell lysates were also plated on 7H10 agar supplemented with 50 µg/ml carbenicillin. This demonstrated that selection of spontaneous β-lactam resistant mutants did not occur during the course of infection. To determine the appropriate carbenicillin concentration necessary to kill intracellular bacteria, THP-1 infection experiments were performed with a range of antibiotic concentrations (Fig. 4b). Carbenicillin at 1 mg/ml was determined to be the lowest concentration of antibiotic that caused optimal killing of sensitive intracellular *M. tuberculosis* and was used in subsequent experiments.

RESULTS

'BlaTEM-1 is exported by Sec and Tat signal sequences in *M. tuberculosis*

β-lactamase is an ideal reporter for protein export because it must be localized beyond the bacterial cytoplasmic membrane to effectively protect the bacterium from β-lactam antibiotics. Therefore, it can be used in protein fusions to identify proteins that are extracytoplasmic. An attractive feature of a β-lactamase reporter is that a selection for β-lactam resistant colonies can be performed, as opposed to a more labor-intensive screen. In the past, we showed that the endogenous β-lactamase of *M. tuberculosis* BlaC can function as a reporter of export exclusively by the Tat pathway when expressed in the β-lactam sensitive *ΔblaC* mutant of *M. tuberculosis* or *ΔblaS* mutant of *M. smegmatis* (McDonough *et al*., 2005). Since the Sec pathway is likely responsible for the majority of protein export in *M. tuberculosis*, we were interested in utilizing a β-lactamase reporter that additionally works with Sec exported proteins. For this reason, we tested the *E. coli* TEM-1 β-lactamase (BlaTEM-1) which has been used in other bacteria to report on proteins exported by Sec, Tat, Type II and Type III secretion systems (Broome-Smith *et al*., 1990; Charpentier & Oswald, 2004; Sauvonnet & Pugsley, 1996; Stanley *et al*., 2002).

A series of multi-copy kanamycin-marked ‘*blaTEM-1* plasmids were constructed and electroporated into the *ΔblaC* mutant of *M. tuberculosis* (Fig. 1). The resulting kanamycin resistant strains were tested for the ability to grow in the presence of 50 μg/ml of the β-lactam carbenicillin. When the truncated '*blaTEM-1* reporter without a signal sequence was expressed in the *ΔblaC* mutant of *M. tuberculosis*, the strain remained carbenicillin-sensitive. In fact, no colonies of the strain expressing the truncated ‘BlaTEM-1 grew on agar containing carbenicillin even after extended incubation (Fig. 1 and Fig 2). However, expression of a hybrid protein comprised of a Sec signal sequence from Mpt63, a well-established secreted protein of *M. tuberculosis* (Horwitz *et al*., 1995;Manca *et al*., 1997), fused to 'BlaTEM-1 (ssMpt63-‘BlaTEM-1) protected the *ΔblaC* mutant from carbenicillin, as was evident by the ability of this strain to grow on carbenicillin agar plates (Fig. 1 and Fig 2) . We similarly tested a fusion protein in which the Sec signal sequence of a proven cell wall-associated lipoprotein, Mpt83 (Hewinson *et al*., 1996), was fused to ‘BlaTEM-1. This construct also conferred β-lactam resistance to *ΔblaC M. tuberculosis* (Fig. 1). Of note, the ssMpt83-‘BlaTEM-1 fusion protein also included the first 31 amino acids of the mature Mpt83 protein as well as the native *mpt83* promoter which is reported to be active at very low levels *in vitro* (Hewinson *et al*., 1996;Said-Salim *et al*., 2006).

Finally, we tested the signal sequence of PlcB, a proven cell wall-associated phospholipase C, for the ability to promote export of enzymatically active ‘BlaTEM-1 (Johansen *et al*., 1996; Raynaud *et al*., 2002). PlcB has a predicted Tat signal sequence, and the ssPlcB-‘BlaTEM-1 fusion also allowed *ΔblaC M. tuberculosis* to grow in the presence of carbenicillin (Fig. 1).

To determine whether the ssPlcB-'BlaTEM-1 fusion was exported by the Tat pathway, it was tested in *ΔblaS M. smegmatis* and in a *ΔtatA ΔblaS M. smegmatis* double mutant (McDonough *et al*., 2005) in two independent experiments. When the ssPlcB-'BlaTEM-1 fusion protein was expressed in *ΔblaS M. smegmatis*, 92% of colonies were carbenicillin resistant. However, when the same construct was expressed in the *ΔtatA ΔblaS* mutant only an average 7% of colonies were carbenicillin resistant indicating that the Tat pathway functions in the export of this fusion protein. To show that a functional Tat pathway was not required for export of the Sec signal sequence-'BlaTEM-1 fusion, we similarly evaluated export of ssMpt63-'BlaTEM-1. When expressed in *ΔblaS* and the *ΔtatA ΔblaS* mutants, ssMpt63-'BlaTEM-1 conferred carbenicillin resistance to 90% and 95% of colonies, respectively. This indicated, as expected, no role for the Tat pathway in exporting a Sec signal sequence-'BlaTEM-1 fusion.

In each example where a *M. tuberculosis* signal sequence (Sec or Tat) was fused to ‘BlaTEM-1, *ΔblaC M. tuberculosis* was protected from β-lactam attack. To demonstrate that the inability of the ‘BlaTEM-1 reporter lacking a signal sequence to protect against carbenicillin was due to lack of export, as opposed to lack of expression, whole cell extracts of ‘BlaTEM-1 expression strains were prepared and assayed for cell-associated β-lactamase. To test for enzyme activity, we used the chromogenic β-lactam nitrocefin, which turns red following cleavage by β-lactamase (O'Callaghan *et al*., 1972). During a 15 minute incubation the nitrocefin was hydrolyzed by all strains expressing ‘BlaTEM-1 constructs, while *ΔblaC M. tuberculosis* demonstrated no activity, similar to PBS alone (data not shown). Importantly, β-lactamase activity was detected with the truncated ‘BlaTEM-1 reporter lacking a signal sequence. In fact, the lysate from the ‘BlaTEM-1 strain converted nitrocefin to the red product almost instantaneously and faster than any other strain tested. We similarly detected β-lactamase activity in whole cell lysates of *ΔblaC M. tuberculosis* expressing the 'BlaC reporter lacking its native signal sequence.

We also compared the level of each ‘BlaTEM-1 fusion protein present in whole cell lysates from the respective *M. tuberculosis* strains by immunoblots with antibodies specific for BlaTEM-1. This revealed a wide variation in the amount of ‘BlaTEM-1 protein produced by the different strains (Fig. 3). The non-exported ‘BlaTEM-1 expressed off the *hsp60* promoter (P_{hsp60}) was the most abundant protein detected. P_{hsp60} is considered a relatively strong promoter and is, therefore, present on many mycobacterial shuttle vectors (Stover *et al*., 1991). In comparison, the P_{hsp60} driven ssPlcB-‘BlaTEM-1 and ssMpt63-‘BlaTEM-1 were expressed at lower levels (59% and 0.9% of the level of the non-exported ‘BlaTEM-1 construct, respectively). Since *mpt83* is expressed at relatively low levels *in vitro* we expected the ssMpt83-‘BlaTEM-1 fusion to be weakly expressed (Hewinson *et al*., 1996;Said-Salim *et al*., 2006;Schnappinger *et al*., 2003). In fact, it was nearly undetectable by immunoblot, present at only 0.4% of the amount of non-exported ‘BlaTEM-1 construct. The bands detected on the immunoblot are in general agreement with the predicted molecular weight of the expressed proteins. ‘BlaTEM-1, lacking a signal sequence, has a predicted size of 28 kDa. Since whole cell lysates were analyzed in these experiments it is possible to see processed protein and/or uncleaved cytosolic precursor, which may explain the larger sized ssPlcB-‘BlaTEM-1 product. The signal sequences of PlcB and Mpt63 would add approximately 3 and 4 kDa, while the Mpt83 signal sequence and fused portion of the mature protein would add approximately 11 kDa, if left intact.

These observations suggested that even though ‘BlaTEM-1 does not promote growth in the presence of carbenicillin, a significant amount of β-lactamase was produced and accumulated within the bacterium. Together, our results indicated that in *ΔblaC M. tuberculosis* 'BlaTEM-1 must be exported to confer protection against β-lactam antibiotics, that β-lactam resistance can be used to report on export, and that this reporter can be exported by Sec or Tat signal sequences and is compatible with different levels of expression.

The *ΔblaC* mutant of *M. tuberculosis* is sensitive to β-lactams during intracellular growth in human THP-1 cells

β-lactam antibiotics can be used for clinical treatment of intracellular pathogens such as *Listeria monocytogenes* (Safdar & Armstrong, 2003), and have been shown to reduce the population of phagocytosed *Staphylococcus aureus* (Barcia-Macay *et al*., 2006). This indicates that β-lactams can enter macrophages and inhibit intracellular growth of some bacteria. The *ΔblaC* mutant of *M. tuberculosis* is sensitive to β-lactams *in vitro*, and we set out to test if this mutation also makes *M. tuberculosis* susceptible to β-lactams during growth in host cells.

Intracellular growth of the *ΔblaC* mutant was not previously evaluated; therefore, we first tested the ability of this mutant to grow within human monocytic THP-1 cells. THP-1 cells were infected at a m.o.i. of 0.1 with either the *ΔblaC* mutant or the virulent parental H37Rv strain. After a four hour period of infection, the THP-1 monolayer was washed to remove non-cell associated bacilli and fresh media was added back. Growth over a five day period was assessed by plating of infected host cell lysates for viable bacilli. The *ΔblaC* mutant showed no difference in intracellular growth when compared to H37Rv (Fig. 4a). Of note, we confirmed that *M. tuberculosis* does not grow in the THP-1 culture medium as previously reported (Zhang *et al*., 1998).

To determine if the *ΔblaC* mutant was sensitive to β-lactams during intracellular growth, THP-1 cells were infected with *ΔblaC M. tuberculosis* and, following the washes to remove extracellular bacilli, media containing different concentrations of carbenicillin was added to the infected monolayers. After five days incubation, the infected monolayers were washed to remove carbenicillin and lysed to plate for viable bacilli. In the absence of carbenicillin, the *ΔblaC* mutant grew in THP-1 cells as previously seen. However, as the concentration of carbenicillin during the intracellular growth period increased, growth of the mutant diminished. At carbenicillin concentrations of ≥ 0.8 mg/ml substantial killing of the mutant was observed (Fig. 4b). These results indicated that the *ΔblaC* mutant is sensitive to β-lactam antibiotics during intracellular growth, and it suggested that the β-lactamase reporters could be used to study protein export during intracellular growth. Additional experiments showed that a concentration of 1 mg/ml carbenicillin was sufficient to achieve significant killing of the *ΔblaC* mutant of *M. tuberculosis* in THP-1 cells, and this concentration was used in all subsequent experiments.

Export of β-lactamase protects intracellular *ΔblaC M. tuberculosis* from β-lactam antibiotics

A reporter system that works with intracellularly growing *M. tuberculosis* would be of great value for identifying exported proteins that are expressed and exported only during infection. Having shown that the *ΔblaC* mutant was sensitive to β-lactams during intracellular growth, we tested if β-lactamase could be used to report on protein export by *M. tuberculosis* growing in host cells. We tested fusion proteins expressing the ‘BlaC and ‘BlaTEM-1 reporters for the ability to protect the *ΔblaC* mutant in β-lactam treated THP-1 cells. In each experiment we compared an exported fusion protein to the truncated reporter alone. To test the ‘BlaC reporter, which works with Tat exported proteins only, THP-1 cells were infected with the *M. tuberculosis ΔblaC* mutant expressing ssPlcB-‘BlaC or ‘BlaC only. Media with or without 1 mg/ml carbenicillin was added and the course of infection was monitored over a five day period. In the absence of carbenicillin, both strains grew in THP-1 cells during the course of the experiment. However, in the presence of carbenicillin, the strain expressing the truncated reporter alone did not grow and was reduced by one log over five days while the strain expressing the exported ssPlcB-‘BlaC fusion protein was protected from carbenicillin and grew normally (Fig. 5a).

The ‘BlaTEM-1 fusions were similarly tested. When THP-1 cells were infected with *ΔblaC M. tuberculosis* expressing either the exported ssMpt63-‘BlaTEM-1 or the ‘BlaTEM-1 reporter alone, only the strain expressing ssMpt63-‘BlaTEM-1 fusion grew in THP-1 cells in the presence of carbenicillin. The non-exported ‘BlaTEM-1 strain was sensitive to the β-lactam and was reduced in number by one log (Fig. 5b). Similarly, *ΔblaC M. tuberculosis* exporting ssMpt83-‘BlaTEM-1 fusion was able to grow in carbenicillin treated THP-1 cells, while the non-exported ‘BlaTEM-1 construct did not confer resistance to the *ΔblaC* mutant (Fig. 5c).

These experiments demonstrated that both the Tat specific ‘BlaC reporter and the more permissive ‘BlaTEM-1 reporter can report on protein export while *M. tuberculosis* is growing in β-lactam treated host cells. The use of β-lactamase reporters with intracellular *M. tuberculosis* represents a powerful tool for the study and identification of proteins exported during growth in host cells.

DISCUSSION

The exported proteins of *M. tuberculosis* have been the subject of research attention for some time. This stems from the well-established fact that the majority of bacterial virulence factors and antigens are proteins exported out of the cytoplasm to the bacterial cell envelope or secreted out from the bacterium (Finlay & Falkow, 1997). In fact, there is a growing list of *M. tuberculosis* exported and secreted proteins shown to contribute to virulence or to development of a host immune response (Kurtz & Braunstein, 2005). Genetic reporters have proven to be powerful tools for identifying these extracytoplasmic proteins. The construction of a β-lactam sensitive *ΔblaC* mutant of *M. tuberculosis* opened the door for using β-lactamases as reporters of protein export directly in *M. tuberculosis*. The 'BlaC reporter can be used as a Tat specific reporter while the 'BlaTEM-1 reporter, shown here, can work with Sec or Tat signal sequences. An advantage of β-lactamase reporters is that they can be used to select for exported fusion proteins, as opposed to more labor intensive screening. In addition, we showed here for the first time that resistance to β-lactam antibiotics can be used to report on protein export during intracellular growth of bacteria. Even in more genetically tractable bacterial pathogens, the identification of proteins exported or secreted from within host cells is a challenge.

Because β-lactams target cell wall modifying enzymes, β-lactamases must be exported in order to protect against these drugs. This export requirement was previously exploited with fusion proteins expressed in *E. coli* and other bacteria grown *in vitro* (Broome-Smith *et al*., 1990; Lee & Hughes, 2006). Here we showed that BlaTEM-1 can also report on protein export directly in *ΔblaC M. tuberculosis*. The three *M. tuberculosis* signal sequences tested in our study are from well-established secreted or cell- wall associated proteins. Mpt63 (Rv1926c, 16kDa protein) has a predicted Sec signal sequence and is one of the four most abundant *M. tuberculosis* proteins secreted into culture media during *in vitro* growth (Horwitz *et al*., 1995). Mpt83 (Rv2873) is a glycosylated lipoprotein (Hewinson *et al*., 1996; Sutcliffe & Harrington, 2004) that is exported to the cell wall of *M. tuberculosis*. Mpt83 has a predicted Sec signal sequence with a lipoprotein signal peptidase (LspA) cleavage site and the requisite conserved cysteine for lipid modification. PlcB (Rv2350c, phospholipase C) is a cell wall associated protein of *M. tuberculosis* shown to function in virulence (Johansen *et al*., 1996; Raynaud *et al*., 2002). Unlike Mpt63 and Mpt83, PlcB has a predicted Tat signal sequence including a twin-arginine motif (Dilks *et al*., 2003). Signal sequences from all three of these proteins were able to promote export of a fused 'BlaTEM-1 reporter on the basis of production of β-lactam resistance. Notably, the ssMpt83-'BlaTEM- 1 fusion protein was expressed from the native *mpt83* promoter and the fusion protein included the predicted signal sequence plus 31 amino acids of the mature Mpt83 protein. This demonstrated the ability of the reporter to work with different strength promoters and extended protein sequences. It is important to note that even though variable levels of fusion protein were detected in *M. tuberculosis* whole cell lysates as determined by immunoblot, each exported fusion provided sufficient protection against 50 µg/ml carbenicillin while the most abundant 'BlaTEM-1 without an export signal did not confer β-lactam resistance.

Previously, we showed that the PlcB signal sequence is able to drive export of functional 'BlaC in a Tat- and twin RR-dependent manner (McDonough *et al*., 2005). In *E. coli* the 'BlaTEM-1 reporter works with both Sec and Tat signal sequences (Broome-Smith *et al*., 1990; Stanley *et al*., 2002). The Sec and Tat pathways appear essential in *M. tuberculosis* (Braunstein *et al*., 2001; Saint-Joanis *et al*., 2006; Sassetti *et al*., 2003). Therefore, to investigate the mode of export of the ssPlcB-'BlaTEM-1 fusion protein it was tested in *M. smegmatis ΔblaS* and in a *M. smegmatis ΔtatA ΔblaS* double mutant. A 93% reduction in β-lactam resistant colonies was observed in the *M. smegmatis ΔtatA ΔblaS* double mutant. Thus, the Tat pathway is involved in the export of ssPlcB-'BlaTEM-1, although other export pathways participate as well. The signal sequence of PlcB may be promiscuous in targeting the Tat or Sec pathway for export depending on the folded or unfolded nature of a fused reporter element. Similar results were recently shown for some predicted Tat signal sequences in *E. coli* (Tullman-Ercek *et al*., 2007).

In addition to working with the Sec and Tat pathways, the‘BlaTEM-1 reporter has been used with type II and type III secretion systems of Gram-negative bacteria (Charpentier & Oswald, 2004; Sauvonnet & Pugsley, 1996). Since substrates of the type III secretion system lack conventional N-terminal signal sequences, it remains possible that the ‘BlaTEM-1 reporter will also work with non-conventional exported proteins of *M. tuberculosis*.

An interesting category of exported proteins that has been largely overlooked are those proteins only expressed and/or exported during the course of infection. We hypothesize that these are proteins exclusively exported in the host environment including virulence factors and protective antigens. Further, only a small number of the exported *M. tuberculosis* proteins identified *in vitro* have ever been directly investigated during intracellular growth in host cells (Kurtz & Braunstein, 2005). For most of these studies, immunomicroscopy was used to localize the proteins in *M. tuberculosis* infected macrophages, which required development of suitable antibodies. We reasoned that if β-lactam antibiotics can reach intracellular *ΔblaC M. tuberculosis*, β-lactamase reporters should additionally work during intracellular growth. β-lactam antibiotics do not normally accumulate in eukaryotic cells; however, antibiotics of this class freely diffuse in and out of host cells (Tulkens, 1991), and β-lactam antibiotics are used to treat some intracellular bacterial infections (Safdar & Armstrong, 2003). More specifically, β-lactams reach intracellular *Staphylococcus aureus* and *Listeria monocytogenes* and prevent growth of these organisms in THP-1 cells (Barcia-Macay *et al*., 2006; Carryn *et al*., 2003). Here we showed that *ΔblaC M. tuberculosis* in THP-1 cells was also susceptible to carbenicillin. Thus, BlaC is responsible for *M. tuberculosis* resistance to β-lactam antibiotics during intracellular growth, indicating that the chromosomal *ΔblaC* is a key factor preventing the use of β-lactams to treat *M. tuberculosis* infection.

When the set of exported β-lactamase fusion proteins was tested for the ability to protect *ΔblaC M. tuberculosis* from β-lactam treatment during intracellular growth, all exported fusions conferred resistance. In contrast, the truncated non-exported β-lactamase reporters were not protective. These experiments demonstrated the effectiveness of both ‘BlaC and ‘BlaTEM-1 reporters to identify *M. tuberculosis* sequences that drive export of each reporter during growth within host cells. Because the ssMpt83-‘BlaTEM-1 fusion was expressed from the native promoter, our results indicate that Mpt83, a protein of unknown function, is expressed and exported during intracellular infection. This result is consistent with the reported induction of *mpt83* in macrophages (Schnappinger *et al*., 2003).

Several approaches have described proteins exported by *M. tuberculosis in vitro*, but a different suite of proteins may be exported during infection of the host. The intracellular β-lactamase reporter system we describe represents a new genetic tool for studying protein export in *M. tuberculosis*. It can be used to directly test the intracellular export of a protein of interest. We also hope to use it in combination with multiple rounds of infection and selection of β-lactam resistant clones from a *M. tuberculosis* fusion library. This should serve to identify the most interesting category of proteins; namely, those that are exported during intracellular growth and missed by alternative methods.

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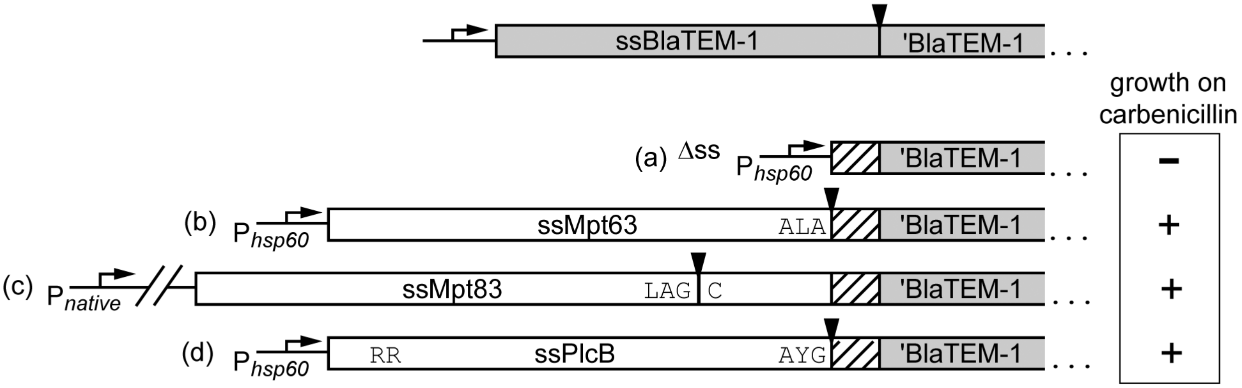

Fig. 1. Schematic representation of signal sequence-'BlaTEM-1 fusion constructs

Mycobacterial shuttle plasmids were designed to encode fusion proteins of *M. tuberculosis* peptide sequence (open boxes) with a truncated 'BlaTEM-1 protein (gray boxes) lacking its native signal sequence. The hatched boxes indicate plasmid-derived peptide sequence that is present as a result of the cloning process. The constructs were driven off the constitutive *M. tuberculosis hsp60* promoter for (a) pJES102/'*blaTEM-1*, (b) pJES103/ss*mpt63-'blaTEM-1*, and (d) pJES101/ss*plcB-'blaTEM-1*. The native *M. tuberculosis* promoter located upstream of the *mpt83* operon was used to drive expression of (c) pJES129/ ss*mpt83-'blaTEM-1* (promoters indicated by arrows). Signal peptidase cleavage sites are indicated by arrowheads and by the AxA/G recognition motif for PlcB and Mpt63, and the LAGC lipobox recognition motif for Mpt83. Diagram not to scale; ss, signal sequence.

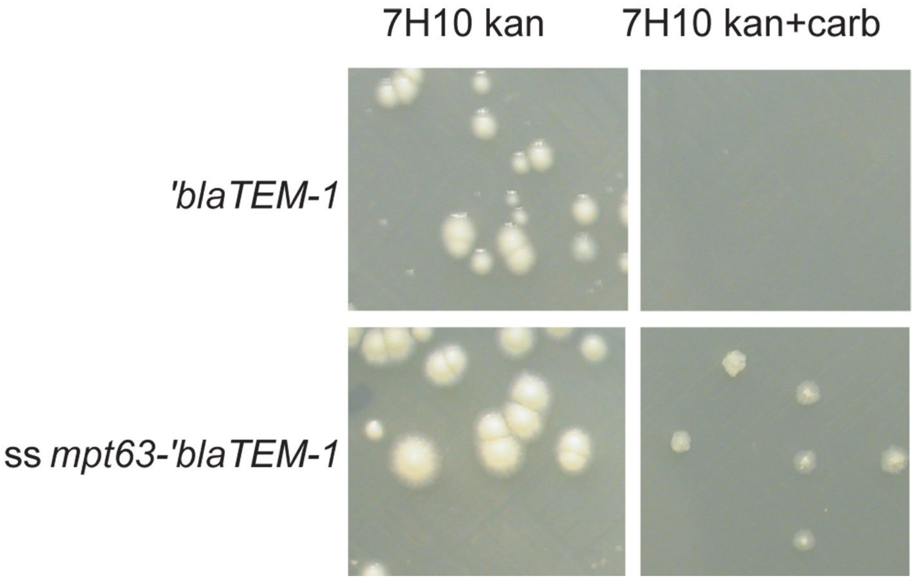

Fig. 2. 'BlaTEM-1 does not provide β-lactam-resistance to *ΔblaC M. tuberculosis*

Plasmids encoding the indicated '*blaTEM-1* fusions were electroporated into *M. tuberculosis ΔblaC*. The resulting strains were then plated on 7H10 plates supplemented with either kanamycin and 0.05% tween or kanamycin and carbenicillin without tween. Plates were inspected for growth following 21–25 days of incubation. Not shown are colonies expressing ssMpt83-‘BlaTEM-1 and ssPlcB-‘BlaTEM-1; growth on plates containing carbenicillin for these strains was similar to that conferred by ssMpt63-‘BlaTEM-1.

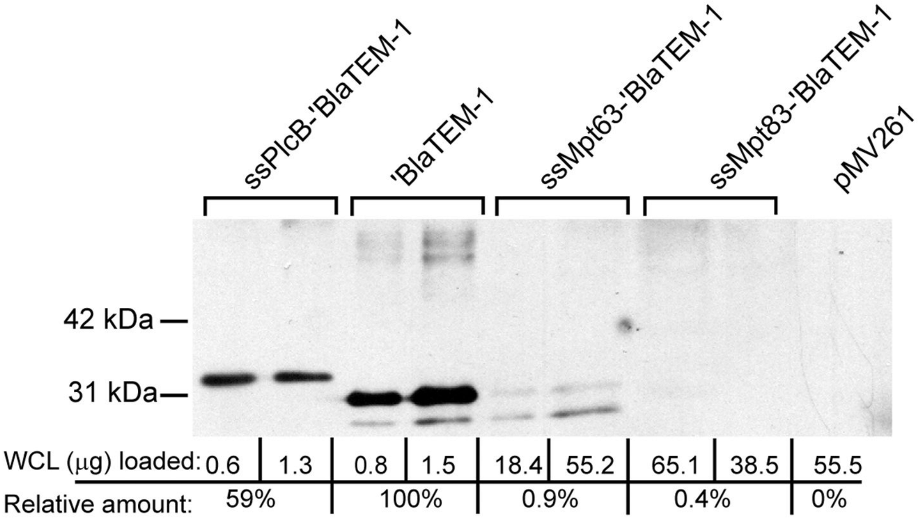

Fig. 3. ‘BlaTEM-1 fusion proteins are detected at different amounts in *M. tuberculosis* whole cell lysates

Protein present in whole cell lysates (WCL) from each of the indicated *ΔblaC* strains were separated by SDS-PAGE and immunoblotted using primary antibody specific for BlaTEM-1. Comparative signal was quantified by measuring pixel density of an equal area for each blotted lysate in duplicate. Average signal intensity per μg of WCL is reported as the amount relative to protein detected in the ‘BlaTEM-1 expressing strain. Due to the different amounts of protein in each strain, it was necessary to load dilutions of the ‘BlaTEM-1 and ssPlcB-‘BlaTEM-1 expressing lysates so that signal from less abundant protein fusions could be simultaneously detected. There was no detectable signal with the WCL from the *ΔblaC* mutant carrying empty pMV261 plasmid.

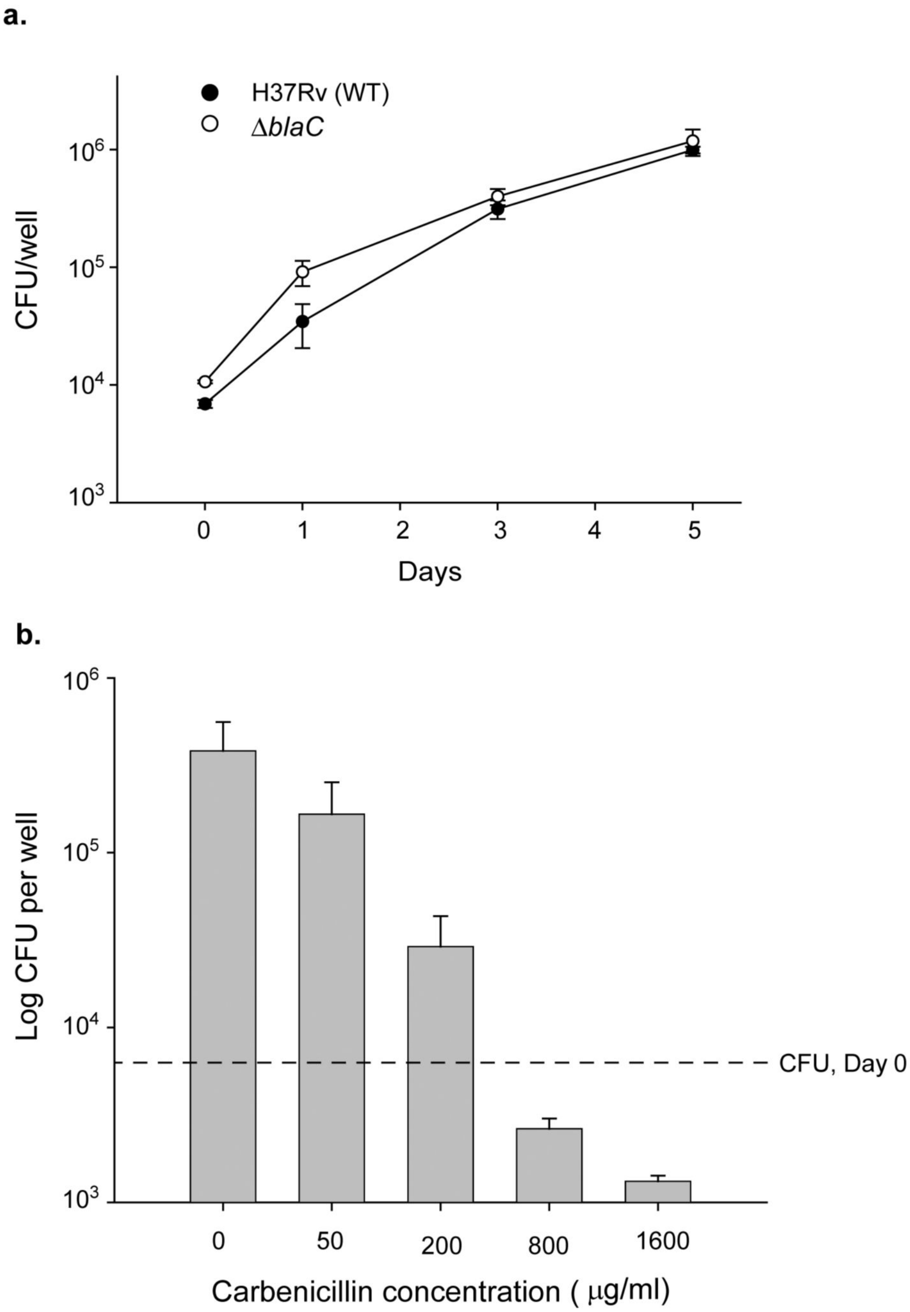

Fig. 4. The *ΔblaC* mutant of *M. tuberculosis* does not have a growth defect and is sensitive to β-lactam antibiotic in human THP-1 macrophage-like cells

(a) THP-1 cells were seeded into 8 well chamber slides, and triplicate wells were infected with either WT H37Rv or *ΔblaC M. tuberculosis* at a m.o.i. of 0.1 bacilli per macrophage. At 4 hours (Day 0), 1, 3 and 5 days post infection, infected wells were washed, lysed, and plated for intracellular bacteria. Error bars represent standard error of the mean of c.f.u. in triplicate wells. (b) THP-1 cells were infected with *ΔblaC M. tuberculosis* as in (a). Following a 4-hour uptake period, wells were washed and indicated concentrations of carbenicillin were added to infected wells. Infected cells were lysed and plated at 4 hours (Day 0) and 5 days post infection to enumerate intracellular bacteria. Dashed line represents average intracellular CFU at 4 hours post infection. Error bars represent standard error of the mean of quadruplicate wells combined from two replicates.

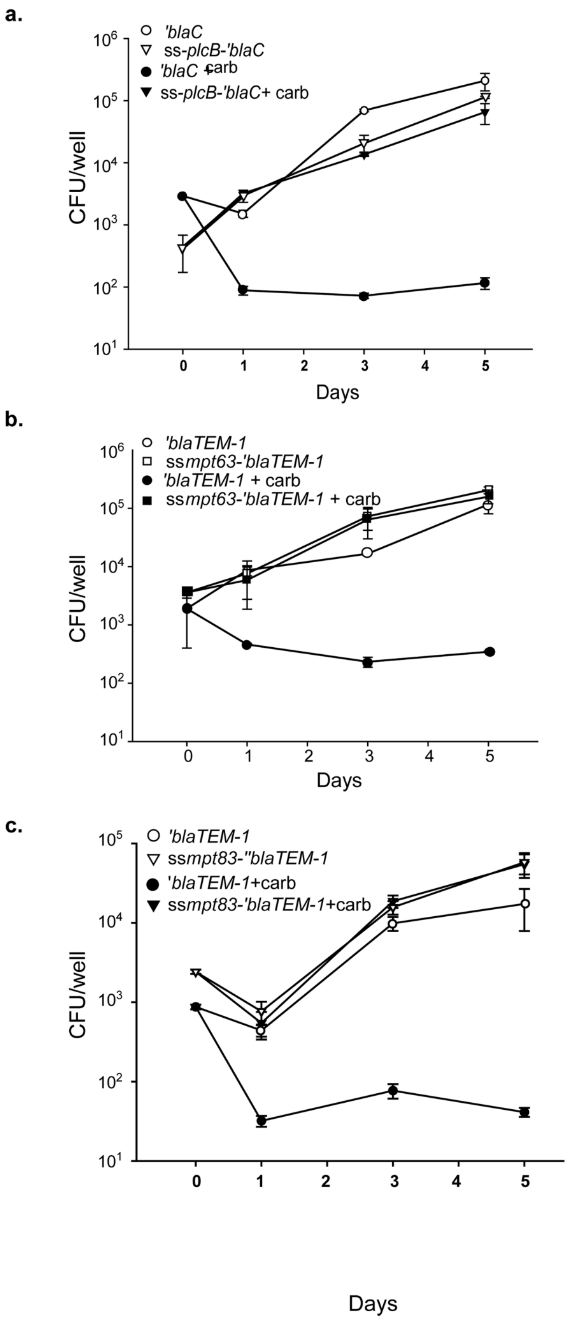

Fig. 5. *M. tuberculosis* signal sequences fused to ‘BlaC and ‘BlaTEM-1 protect intracellular bacilli from β-lactam antibiotics

THP-1 macrophage like cells were infected in triplicate wells with (**a**) *M. tuberculosis ΔblaC* expressing either ssPlcB-‘BlaC or ‘BlaC, (**b**) *M. tuberculosis ΔblaC* expressing either ‘BlaTEM-1 or ssMpt63‘BlaTEM-1 or (**c**) M. tuberculosis blaC expressing ‘BlaTEM-1or ssMpt83-‘BlaTEM-1. Infected cells were then left untreated or treated with 1 mg/ml carbenicillin (carb). Wells were washed, lysed and plated 4 hours (d 0), 1, 3 and 5 days post infection. Each experiment was replicated 3 times in the case of (a) and (b), and 4 times in (c), with similar results.

Table 1

Plasmids used in this study

Plasmid	Genotype	Description	Source
pCC1	*cat oriV ori2*	CopyControl (single copy) blunt cloning vector	Epicentre
pCR2.1	*bla aph* ColE1	TA cloning vector	Invitrogen
pMV261.*kan*	*aph* P*hsp60 oriM* ColE1	Multicopy mycobacterial shuttle plasmid	(Stover *et al.*, 1991a)
pMB219	*aph oriM* ColE1	Multicopy mycobacterial shuttle plasmid	This work
pMB222	*aph* P*hsp60*-ss*plcB* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis plcB* signal sequence in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pMB227	*aph* P*hsp60*-ss*mpt63* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis mpt63* signal sequence in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pMB228	*aph* P*hsp60*-ss*mpt63*-*‘blaC* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis ssmpt63*-*‘blaC* in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pJM109	*aph* P*hsp60*-ss*fbpB*-*‘blaC* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis ssfbpB*-*‘blaC* in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pJM111	*aph* P*hsp60*-ss*plcB*-*‘blaC* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis ssplcB*-*‘blaC* in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pJM113	*aph* P*hsp60*-*‘blaC* (*M. tuberculosis*) *oriM* ColE1	*M. tuberculosis ‘blaC* (no signal sequence) in pMV261 under control of *hsp60* promoter	(McDonough *et al.*, 2005)
pJM114	*cat oriV ori2*	*E. coli ‘blaTEM-1* cloned into pCC1	This work
pJES101	*aph* P*hsp60*-ss*plcB*-*‘blaTEM-1* (*E. coli*) *oriM* ColE1	’*blaTEM-1* from pJM114 cloned into pMB222	This work
pJES102	*aph* P*hsp60*-*‘blaTEM-1* (*E. coli*) *oriM* ColE1	*blaTEM-1* from pJM114 cloned into pMV261	This work
pJES103	*aph* P*hsp60*-ss*mpt63*-*‘blaTEM- 1* (*E. coli*) *oriM* ColE1	’*blaTEM-1* from pJM114 cloned into pMB227	This work
pJES125	*bla aph* ColE1	*M. tuberculosis mpt83* signal sequence and upstream sequence cloned into pCR2.1	This work
pJES128	*aph oriM* ColE1	*blaTEM-1* from pJM114 cloned into *Bam*HI-linearized pMB219	This work
pJES129	*aph* P*mpt83*-ss*mpt83*-*‘blaTEM- 1* (*E. coli*) *oriM* ColE1	*M. tuberculosis mpt83* signal sequence and upstream sequence from pJES125 cloned into pJES128	This work