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Genetic analysis of the β-lactamases of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* and susceptibility to β-lactam antibiotics

Anthony R. Flores,[1] Linda M. Parsons[2] and Martin S. Pavelka, Jr[1]

[1]University of Rochester School of Medicine and Dentistry and Department of Microbiology and Immunology, Rochester, NY 14642, USA

[2]The Wadsworth Center, New York State Department of Health, Albany, NY 12201, USA

Correspondence
Martin S. Pavelka, Jr
Martin_Pavelka@urmc.rochester.edu

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Mycobacteria produce β-lactamases and are intrinsically resistant to β-lactam antibiotics. In addition to the β-lactamases, cell envelope permeability and variations in certain peptidoglycan biosynthetic enzymes are believed to contribute to β-lactam resistance in these organisms. To allow the study of these additional mechanisms, mutants of the major β-lactamases, BlaC and BlaS, were generated in the pathogenic *Mycobacterium tuberculosis* strain H37Rv and the model organism *Mycobacterium smegmatis* strain PM274. The mutants *M. tuberculosis* PM638 (Δ*blaC1*) and *M. smegmatis* PM759 (Δ*blaS1*) showed an increase in susceptibility to β-lactam antibiotics, as determined by disc diffusion and minimal inhibitory concentration (MIC) assays. The susceptibility of the mutants, as assayed by disc diffusion tests, to penicillin-type β-lactam antibiotics was affected most, compared to the cephalosporin-type β-lactam antibiotics. The *M. tuberculosis* mutant had no detectable β-lactamase activity, while the *M. smegmatis* mutant had a residual type 1 β-lactamase activity. We identified a gene, *blaE*, encoding a putative cephalosporinase in *M. smegmatis*. A double β-lactamase mutant of *M. smegmatis*, PM976 (Δ*blaS1*Δ*blaE*::*res*), had no detectable β-lactamase activity, but its susceptibility to β-lactam antibiotics was not significantly different from that of the Δ*blaS1* parental strain, PM759. The mutants generated in this study will help determine the contribution of other β-lactam resistance mechanisms in addition to serving as tools to study the biology of peptidoglycan biosynthesis in these organisms.

INTRODUCTION

The mycobacteria consist of pathogens responsible for significant morbidity and mortality throughout the world, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, as well as opportunistic pathogens, such as *Mycobacterium avium*, *Mycobacterium fortuitum* and *Mycobacterium smegmatis*. It is estimated that one-third of the world population, approximately 2 billion people, are latently infected with *M. tuberculosis*, and that more than 2 million people die each year from tuberculosis (Dye *et al.*, 1999). Interest in the treatment of tuberculosis has grown in recent years due to the increased prevalence of *M. tuberculosis* in the immunocompromised (e.g. AIDS patients) and the emergence and spread of multi-drug-resistant strains.

Abbreviations: ATM, aztreonam; CLA, clavulanic acid; LOT, cephalothin; PBP, penicillin-binding protein; PEN, benzylpenicillin.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are AY332268 and AY442183.

The β-lactam class of antibiotics has not been used in the treatment of *M. tuberculosis* or other mycobacterial infections as mycobacteria are resistant to these antibiotics and produce β-lactamases (Kasik, 1979; Kwon *et al.*, 1995). However, the effectiveness of β-lactam/β-lactamase inhibitor combinations has been shown *in vitro* for *M. tuberculosis* (Chambers *et al.*, 1995; Cynamon & Palmer, 1983; Segura *et al.*, 1998; Sorg & Cynamon, 1987), *M. avium* (Casal *et al.*, 1987), *M. fortuitum* (Utrup *et al.*, 1995; Wong *et al.*, 1988) and *M. smegmatis* (Yabu *et al.*, 1985). Clinical evidence suggests that β-lactam antibiotics in combination with β-lactamase inhibitors may be useful in the treatment of *M. tuberculosis* infection (Chambers *et al.*, 1998; Nadler *et al.*, 1991).

The major β-lactamase, BlaA, of the avirulent *M. tuberculosis* strain H37Ra, has been described in both biochemical and molecular terms and is identical to BlaC, found in the virulent *M. tuberculosis* strain H37Rv (Hackbarth *et al.*, 1997; Voladri *et al.*, 1998). This *M. tuberculosis* β-lactamase bears significant homology to molecular class A β-lactamase

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enzymes; functionally, it appears to be a penicillinase or type 2b β-lactamase (Ambler, 1980; Ambler *et al.*, 1991; Bush *et al.*, 1995). A recombinant form of the *M. tuberculosis* H37Rv BlaC enzyme has been described biochemically (Voladri *et al.*, 1998). However, direct genetic studies of BlaC in *M. tuberculosis* H37Rv are lacking. In addition, a minor β-lactamase having predominantly cephalosporinase activity has been described in *M. tuberculosis* H37Ra (Voladri *et al.*, 1998), but its role in the resistance of *M. tuberculosis* to β-lactam antibiotics is not understood and no gene has been identified.

The major β-lactamase in *M. smegmatis* mc^2155 has been biochemically described and is similar to BlaF, the well-studied molecular class A β-lactamase from *M. fortuitum* (Kaneda & Yabu, 1983; Quinting *et al.*, 1997). A recent report identified a gene, designated *blaA*, encoding the major β-lactamase in *M. smegmatis* (Li *et al.*, 2004), which is the same gene we previously designated *blaS* and describe in this work (A.R. Flores & M. S. Pavelka, Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother. Abstr. 674, 2003). Biochemical studies have revealed the presence of a cephalosporinase in *M. smegmatis* SN_2, which, from inhibitor and substrate profiles, appears to be a functional group 2e β-lactamase (Basu *et al.*, 1997). However, an N-terminal sequence from the purified β-lactamase bears significant homology to class C or functional group 1 enzymes (Basu *et al.*, 1997).

Resistance to β-lactam antibiotics in mycobacteria is generally believed to result from the following mechanisms, singly or in combination: (1) enzymic inactivation by β-lactamases, (2) exclusion of the drug from the site of action by an impermeable cell envelope, and (3) the susceptibility of the target penicillin-binding proteins (PBPs) to inhibition. Drug export pumps may contribute to resistance, but the influence of these pumps appears to be limited (Li *et al.*, 2004). The presence of β-lactamases in these organisms complicates the study of the other β-lactam resistance mechanisms and also interferes with the use of β-lactam antibiotics in the study of peptidoglycan biosynthesis. Here, we have used a genetic approach to study the contribution of the β-lactamases to β-lactam antibiotic resistance in *M. tuberculosis* H37Rv and *M. smegmatis* mc^2155. We identified the major β-lactamase gene, *blaS*, and the minor β-lactamase, *blaE*, in the genome of *M. smegmatis*. The β-lactamase of *M. tuberculosis* (*blaC*), and those of *M. smegmatis* (*blaS* and *blaE*), were deleted by allelic exchange. The resulting mutants were significantly more susceptible to β-lactam antibiotics and had reduced or non-detectable β-lactamase activities; however, the susceptibility of the mutants to penicillin-type β-lactam antibiotics was affected most, compared to the cephalosporin-type β-lactam antibiotics. These mutants will serve as tools for the study of other β-lactam resistance mechanisms and of the interaction of β-lactam antibiotics with the peptidoglycan biosynthesis machinery of *M. tuberculosis* and *M. smegmatis*.

Table 1. Strains used in this study

Strain	Description	Source or reference
E. coli		
DH10B	F^-, *mcrA* Δ(*mrr-hsdRMSmcrBC*) φ80d*lacZ* ΔM15 Δ*lacX74 deoR recA1 ara* Δ*139* (*ara, leu*)7697 *galU galK* λ^-, *rpsL endA1 nupG*	Gibco-BRL
M. smegmatis		
PM274	*ept-1* Δ*lysA4 rpsL6*	Consaul *et al.* (2003)
PM759	PM274 Δ*blaS1*	This work
PM791	PM759 *attB*::pMP283	This work
PM876	PM759 *attB*::pMV361.*hyg*	This work
PM939	PM274 Δ*blaS1* Δ*blaE1*::*res-aph-res*	This work
PM976	PM274 Δ*blaS1* Δ*blaE1*::*res*	This work
M. tuberculosis		
H37Rv	Virulent	AECOM*
PM638	H37Rv Δ*blaC1*	This work
PM669	PM638 *attB*::pMP199	This work
PM670	PM638 *attB*::pMV361.*hyg*	This work

*AECOM, Albert Einstein College of Medicine.

METHODS

Bacterial strains and growth conditions. The strains used in this study are shown in Table 1. *M. tuberculosis* H37Rv is a virulent laboratory strain. *M. smegmatis* PM274 is a lysine auxotroph of the common laboratory strain *M. smegmatis* mc^2155 (Consaul *et al.*, 2003). *Escherichia coli* cultures were grown in Luria–Bertani (LB) broth (Difco; BD Biosciences) or on LB agar. Mycobacterial cultures were grown in Middlebrook 7H9 broth (Difco; BD Biosciences) or on Middlebrook 7H11 medium (Difco; BD Biosciences). All Middlebrook media were supplemented with 0·05 % Tween 80, 0·2 % glycerol (v/v) and ADS (0·5 % BSA, fraction V, 0·2 % glucose and 0·85 % NaCl). Sucrose was added to media at a concentration of 2 %. L-Lysine was added to all *M. smegmatis* cultures at 40 μg ml^{-1}. When necessary, ampicillin (50 μg ml^{-1}; Sigma-Aldrich Chemical) and hygromycin (50 μg ml^{-1}; Roche Applied Science) were added to media. *M. smegmatis* plates were incubated for 3–5 days, while *M. tuberculosis* plates were incubated for 3–4 weeks at 37 °C. Inoculation and growth conditions for allelic exchange in *M. smegmatis* and *M. tuberculosis* were as previously described (Pavelka & Jacobs, 1999).

Antibiotics. The antibiotics used in minimal inhibitory concentration (MIC) determinations, amoxicillin, ampicillin, carbenicillin, cefoxitin, ceftriaxone and cephalothin, were obtained from Sigma-Aldrich, while clavulanic acid was kindly provided by GlaxoSmithKline. Sensi-Discs (BBL; BD Biosciences) used in this study were ampicillin (10 μg), amoxicillin/clavulanic acid (20 μg/10 μg), carbenicillin (100 μg), cefazolin (30 μg), cefixime (5 μg), cefoperazone (75 μg), cefoxitin (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), imipenem (10 μg), isoniazid (5 μg), mezlocillin (75 μg), oxacillin (10 μg), piperacillin (100 μg) and rifampicin (5 μg). In addition, the antibiotics amoxicillin, ampicillin and cefoxitin were tested at a higher concentration by spotting of the appropriate amount of antibiotic on a sterile paper disc (BBL; BD Biosciences) from a stock antibiotic solution.

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Table 2. Plasmids used in this study

Plasmid	Description	Source or reference
pKSI+	Amp^r; high-copy-number cloning vector	Stratagene
pGH542	Mycobacterial shuttle vector containing a γδ-resolvase	G. Hatfull
pMV361.*hyg*	Hyg^r; mycobacterial integrative expression vector	AECOM*
pYUB638	1·4 kb *Mlu*I *res-aph-res* cassette cloned into *Mlu*I site of pKSI+::*lysA*	Pavelka & Jacobs (1999)
pYUB657	Amp^r Hyg^r; suicide vector containing counterselectable marker *sacB*	Pavelka & Jacobs (1999)
MTCY49	*M. tuberculosis* H37Rv sequencing cosmid	Cole *et al.* (1998)
pMP159	6·5 kb *Not*I fragment containing *blaC*$^+$ from MTCY49 inserted into pKSI+ *Not*I site	This work
pMP179	pKSI+::Δ*blaC1*; generated from pMP159 via inverse PCR	This work
pMP180	5·9 kb *Not*I fragment from pMP179 containing Δ*blaC1*, Klenow polymerase treated, and inserted into *Eco*RV site of pYUB657	This work
pMP199	1·0 kb *blaC*$^+$ PCR fragment directionally cloned into *Cla*I/*Hpa*I site of pMV361.*hyg*	This work
pMP222	3·0 kb *blaS*$^+$ PCR fragment from mc^{2}155 genomic DNA inserted into *Xma*I/*Xho*I sites of pKSI+	This work
pMP225	pKSI+::Δ*blaS1*; generated from pMP222 via inverse PCR	This work
pMP252	2·1 kb *Xho*I–*Xba*I fragment from pMP225 containing Δ*blaS1*, Klenow polymerase treated, and inserted into *Eco*RV site of pYUB657	This work
pMP283	1·7 kb *Cla*I–*Sma*I fragment from pMP222 containing *blaS*$^+$ inserted into *Cla*I/*Hpa*I site of pMV361.*hyg*	This work
PMP295	6·7 kb PCR fragment containing *blaE* inserted into the *Xho*I/*Not*I sites of pKSI+	This work
PMP307	pKSI+::Δ*blaE1* generated from pMP295 via inverse PCR	This work
pMP330	pKSI+::Δ*blaE1*::*res-aph-res*	This work
pMP332	6·3 kb fragment containing Δ*blaE1*::*res-aph-res* excised from pMP330 and inserted into the *Eco*RV site of pYUB657	This work

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DNA manipulation. Basic DNA methods were essentially as described by Maniatis *et al.* (1982). Plasmids used in this study are listed in Table 2. Plasmids were constructed in *E. coli* DH10B and were prepared by an alkaline lysis protocol or by Qiagen columns, if used for recombination. DNA fragments were isolated using agarose gel electrophoresis and absorption to a silica matrix (GeneClean; Bio 101), or by QIAquick spin columns (Qiagen). Genomic DNA was prepared as described previously for *M. tuberculosis* (Pavelka & Jacobs, 1999) and *M. smegmatis* (Jacobs *et al.*, 1991). Southern blotting was done as described previously (Pavelka & Jacobs, 1996). Oligonucleotides for PCR were generated by Invitrogen Life Technologies. All restriction and DNA modifying enzymes were from Fermentas or New England Biolabs. Electroporation of *M. smegmatis* and *M. tuberculosis* was as previously described (Pavelka & Jacobs, 1999).

PCR and cloning of *M. smegmatis* and *M. tuberculosis* β-lactamases. The oligonucleotide primers Pv152 (5′-CGTGGT-GCTCGAGGAAATCGC-3′) and Pv153 (5′-AGCCCGAGTACTCG-CGGATG-3′) were used to amplify the *blaS* coding region, including 1 kb of flanking DNA on each side of the gene, from *M. smegmatis* mc^{2}155 genomic DNA (http://www.tigr.org/tdb/mdb/mdbinprogress.html) using *Pfu* polymerase (Stratagene). PCR was performed in a Perkin-Elmer GeneAmp 2400 temperature cycler (Applied Biosystems) with the following programme: 94 °C for 45 s, 1 cycle; 94 °C for 45 s and 60 °C for 4 min, 30 cycles; 72 °C for 10 min. The resulting 2·8 kb PCR fragment was digested with *Xma*I and *Xho*I and ligated into pKSI+ digested with the same enzymes to generate pMP222.

Similarly, *blaE* and adjacent sequence was amplified from *M. smegmatis* mc^{2}155 genomic DNA (http://www.tigr.org/tdb/mdb/mdbinprogress.html) using the oligonucleotide pair Pv187 (5′-CCGAAGGACAT-CTCGAGTCGTTGCGGTTCG-3′) and Pv188 (5′-GCGGACCTCT-CGAGAGCACGCTTGTCATCG-3′) and *Pfu* polymerase. The 6·7 kb product was subsequently digested with the restriction endonucleases *Xho*I and *Not*I and inserted into the same sites of pKSI+ to generate pMP295.

The *M. tuberculosis* H37Rv *blaC* gene was cloned from cosmid MTCY49 of the *M. tuberculosis* H37Rv genome sequencing project (Cole *et al.*, 1998). We excised a 6·5 kb fragment containing *blaC* from MTCY49 using the restriction endonuclease *Not*I and inserted it into the *Not*I site of pKSI+, to generate pMP159.

Construction of suicide plasmids. Construction of suicide plasmids was done essentially as described (Pavelka & Jacobs, 1999). For *blaC* and with pMP159 as a template, the oligonucleotide primer pair Pv135 (5′-GTCACGGAGCTAGCCATTGCCATCGCT-ACCAGCAGTTC-3′) and Pv136 (5′-CGGGCACTGCTAGCGATT-GGATGGCGCGCAACACCACC-3′) was used for inverse XL-PCR with r*Tth* DNA polymerase (Applied Biosystems) in a Perkin-Elmer GeneAmp 2400 temperature cycler with the following parameters: 94 °C for 5 min, 1 cycle; 94 °C 1 min and 60 °C 10 min, 16 cycles; 94 °C 1 min and 60 °C 10 min with time increasing by 15 s for each cycle, 12 cycles; 72 °C for 30 min. The PCR product was purified, digested with *Nhe*I, and self-ligated to generate pMP179. The result was an in-frame 615 bp deletion in the ORF of *blaC* marked with an *Nhe*I restriction site. A *Not*I digest of pMP179 liberated the 5·9 kb fragment containing Δ*blaC1*, which was subsequently treated with Klenow polymerase and ligated into the *Eco*RV site of pYUB657 to generate the suicide plasmid pMP180.

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The plasmid pMP222, containing wild-type *blaS*, was used as template in an inverse PCR reaction (*Pfu* polymerase; Stratagene) with primers Pv176 (5′-GGCGACTACGTATCCACCAACGATGTG-3′) and Pv177 (5′-GGGATCTACGTAGACACGATCGTCCAGC-3′) in a Perkin-Elmer GeneAmp 2400 temperature cycler with the following parameters: 94 °C for 45 s, 1 cycle; 94 °C for 45 s and 63 °C for 7 min, 30 cycles; 72 °C for 10 min. The PCR product was purified, digested with *Sna*BI and self-ligated to generate pMP225. The result was an in-frame, 426 bp deletion in the open reading frame of the *M. smegmatis blaS* gene marked by a *Sna*BI restriction site The 2·0 kb Δ*blaS1* fragment was excised from pMP225 using *Xho*I and *Xba*I, treated with Klenow polymerase, and ligated into *Eco*RV-digested pYUB657 to yield the suicide plasmid pMP252.

An in-frame deletion allele of *blaE* was generated using inverse PCR with pMP295 as a template. *Pfu* polymerase and the primer pair Pv199 (5′-CGGCTATTACTACGTAGGCCCGATGG-3′) and Pv200 (5′-CGGTGAATGTCTTGCTTACGTAGCCGA-3′) generated an in-frame deletion of 591 bp in the ORF of *blaE*. Initial attempts to generate an unmarked deletion of *blaE* were unsuccessful; therefore, the Δ*blaE1* allele was marked using a resolvable kanamycin resistance cassette to ensure a definitive phenotype upon exchange with the wild-type *blaE*. The cassette was excised from pYUB638 (Pavelka & Jacobs, 1999) using *Mlu*I and inserted into a *Sna*BI site of Δ*blaE1* of pMP307 to generate pMP330. Finally, a 6·3 kb fragment containing Δ*blaE1*::*res-aph-res* was excised from pMP330 using *Pvu*II and inserted into the *Eco*RV site of pYUB657 to generate pMP332.

Resolution of Δ*blaE1*::*res-aph-res* using γδ-resolvase. The *res-aph-res* cassette was resolved in strain PM939 (Δ*blaS1* Δ*blaE1*::*res-aph-res*) using the γδ-resolvase supplied *in trans* on a mycobacterial shuttle vector (pGH542; a gift from Graham Hatfull, University of Pittsburgh) to yield strain PM976 (Δ*blaS1* Δ*blaE1*::*res*). The resulting allele, Δ*blaE1*::*res*, contains an out-of-frame insertion in the Δ*blaE1*-coding region.

Antimicrobial susceptibility testing. Zones of inhibition measured by disc diffusion (Sensi-Disc) and MICs were used to determine changes in antibiotic susceptibility in the β-lactamase mutants. The procedure used for disc diffusion in *M. tuberculosis* and *M. smegmatis* was as follows. *M. tuberculosis* cultures were grown to approximately mid to late exponential phase in 10 ml Middlebrook 7H9. Hygromycin was added for PM669 and PM670. Cells were pelleted, washed once in fresh medium, and resuspended in 10 ml fresh medium. Then, 150 μl of washed culture was spread on Middlebrook 7H11 and the antibiotic Sensi-Disc was placed in the centre. Plates were incubated for 2 weeks and zones of inhibition measured to the nearest 5 mm. *M. smegmatis* cultures were grown to mid-exponential phase (OD_{600} 0·4–0·6) in 10 ml Middlebrook 7H9. Hygromycin was added to PM791 and PM876 cultures. Cells were washed once in fresh medium, and resuspended in an equal volume. A pour-plate technique was used by adding 200 μl washed cells to 3·5 ml molten top agar (0·6 % agarose, 0·2 % glycerol, v/v) and pouring onto Middlebrook 7H11 plates. Sensi-Discs were placed in the centre and the plates were incubated for 2–3 days. Zones of inhibition were measured to the nearest 1 mm.

M. tuberculosis MICs were determined at The Wadsworth Center using the radiometric (BACTEC) method. The source of the inoculum was freshly grown *M. tuberculosis* from a primary 7H12 liquid medium (BACTEC TB vial) with a GI (growth index) reading between 900 and 999. After vortexing and passing the suspension through a syringe to break up clumps of bacteria, 0·1 ml of the suspension was used to inoculate vials containing various concentrations of drugs and a control vial (C-0) without any drug. A 1 : 100 dilution of the suspension was used to inoculate a second control vial (C-100). When the growth in the C-100 vial, inoculated with 1 % of the inoculum in the drug-containing vials, reached a GI of 30, it was used to compare increases in daily readings of the drug-containing vials.

M. smegmatis MICs were determined using a broth macrodilution method (Jorgensen *et al.*, 1999). Briefly, cultures were harvested at mid-exponential phase (OD_{600} 0·4–0·6), washed once in fresh media, and diluted 100-fold. The diluted culture was used to inoculate 4 ml media containing serially diluted antibiotic (approx. 10^5 c.f.u. per tube). The tubes were incubated for 3 days on a rotary drum at 37 °C. The MIC was determined to be the lowest concentration at which no growth was observed after 3 days incubation.

Whole-cell lysates. *M. smegmatis* lysates were obtained by French press (Aminco). Cells from saturated cultures ($OD_{600} \geqslant 1·0$) were pelleted, washed twice in cold buffer (1 × PBS, pH 7·0), and subsequently resuspended in 3 ml buffer with DNase (100 U; Roche Applied Science), RNase A (100 μg; Sigma-Aldrich) and protease inhibitor [3 mM 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF); Calbiochem] added. Cells were broken in a French pressure cell [14 000 p.s.i. (96·6 MPa); four applications] and cell debris removed by centrifugation (12 000 ***g***; 30 min). Sterile *M. tuberculosis* lysates were obtained using a FastPrep instrument (Qbiogene) and FastPROTEIN Blue lysing matrix. Control experiments using *M. smegmatis* cells showed that lysate preparation using the FastPrep machine was as efficient as using the French pressure cell. Cells were pelleted from 50 ml saturated cultures, washed twice in cold buffer (1 × PBS, pH 7·0) and resuspended in 5 ml buffer. DNase, RNase A and protease inhibitor were added as for *M. smegmatis*, above. Resuspended cells were distributed to lysing matrix tubes (1 ml each) and subjected to two disruptions at a speed setting of 6·0 for 30 s. The cells were chilled on ice for 5 min between disruptions. Lysate and lysing matrix were transferred to a 15 ml conical tube and the debris was pelleted (4700 ***g***, 10 min). The resulting lysate was filtered twice through a 0·2 μm syringe filter. Control experiments using *M. smegmatis* lysates showed that filtration of lysates does not affect the detection of β-lactamase activity. Protein concentration of whole-cell lysates was determined using the Bradford method (Bio-Rad).

Nitrocefin assays. The chromogenic cephalosporin nitrocefin (Oxoid) was used to assay β-lactamase activity in whole-cell lysates (O'Callaghan *et al.*, 1972). We empirically determined the amount of total lysate protein to add to achieve a linear response. Assays were performed at 22 °C with 100 μM nitrocefin in 1 × PBS, pH 7·0. Hydrolysis was monitored at 486 nm using a Beckman DU530 spectrophotometer (Beckman Instruments) and absorbance recorded every 30 s for 15 min. The amount of nitrocefin hydrolysed per unit time was determined using Beer's law and the molar extinction coefficient of nitrocefin, at 486 nm, of 20 500. Finally, a slope was calculated to estimate the amount of nitrocefin hydrolysed per min. The rate of nitrocefin hydrolysis for each strain was expressed as micrograms of nitrocefin hydrolysed per minute per milligram total lysate protein.

For inhibition and competition assays with *M. smegmatis* lysates, the β-lactam antibiotics aztreonam (ATM; ICN Pharmaceuticals), clavulanic acid (CLA), benzylpenicillin (PEN; ICN Pharmaceuticals) or cephalothin (LOT; Sigma-Aldrich) were added prior to each assay and nitrocefin hydrolysis was determined as described above.

Nucleotide sequence accession numbers. The DNA sequence of a 2482 bp PCR product containing the *blaS* ORF was submitted to GenBank and given the accession number AY332268. The DNA sequence of a 2133 bp fragment containing the *blaE* ORF was submitted to GenBank and given the accession number AY442183.

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RESULTS

Identification of the *M. smegmatis* major and minor β-lactamases

We used the mycobacterial β-lactamase gene *blaF* from *M. fortuitum* (GenBank accession no. AAA19882) to search the unfinished *M. smegmatis* mc^{2}155 genome (The Institute for Genomic Research, TIGR; http://www.tigr.org/tdb/mdb/mdbinprogress.html), and retrieved a gene (we term *blaS*) whose product was 70 % homologous to BlaF. An alignment of this *M. smegmatis* β-lactamase, BlaS, the *M. fortuitum* BlaF, and the BlaC β-lactamase from *M. tuberculosis* H37Rv (GenBank accession no. NP_216584) is shown in Fig. 1.

Another putative β-lactamase gene, that we designate *blaE*, was identified in the *M. smegmatis* mc^{2}155 unfinished genome using the previously reported N-terminal sequence of a purified cephalosporinase from *M. smegmatis* SN_2 (Basu *et al.*, 1997). Fig. 2 shows an alignment of the *M. smegmatis* BlaE protein and known class C β-lactamases.

Construction of deletion mutants

Mutants with deletions of the β-lactamase genes were constructed by a previously described methodology using a mycobacterial suicide vector containing the counter-selectable marker *sacB* (Pavelka & Jacobs, 1999). Southern hybridizations (data not shown) verified the gene replacements in the mutants used in this study, PM638 (*M. tuberculosis* Δ*blaC1*), PM759 (*M. smegmatis* Δ*blaS1*) and PM976 (*M. smegmatis* Δ*blaS1*Δ*blaE1*::*res*).

Antimicrobial susceptibility testing

Susceptibility testing for both *M. smegmatis* and *M. tuberculosis* included disc diffusion using Sensi-Discs. This method was chosen for the initial screening of the allelic exchange mutants because of ease of use and also because more antibiotics are readily available as Sensi-Discs than are available in powder form. This disc diffusion method has been used in the past for susceptibility determinations in fast-growing (Cynamon & Patapow, 1981; Wallace *et al.*, 1979) and slow-growing (Jarboe *et al.*, 1998) mycobacteria.

Results from disc diffusion experiments for the *M. smegmatis blaS* deletion mutant are shown in Table 3. The parental strain, PM274, was not susceptible to β-lactam antibiotics, with the exception of cefoxitin and imipenem. As expected, PM274 was susceptible to amoxicillin in the presence of the β-lactamase inhibitor clavulanic acid. For the *M. smegmatis* Δ*blaS1* mutant PM759, increased susceptibility to β-lactam antibiotics was evident from the appearance of zones of growth inhibition. This phenotype was observed for the mutant with all β-lactam antibiotics except oxacillin, ceftriaxone and cefixime. Note that there was no increase in susceptibility to cefoxitin and imipenem and that the presence of the β-lactamase inhibitor clavulanic acid did not affect the susceptibility of the mutant to amoxicillin. We also observed no change in susceptibility between the parental strain and the mutant

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BlaS MTN--LSRRSVLIGSLAVMAAAGVR--MPTAS--------AAPVDDRIADLERRNNASIG
BlaF MTG--LSRRNVLIGSLVAAAAVGAG--VGGAAPA-----FAAPIDDQLAELERRDNVLIG
BlaC MRNRGFGRRELLVAMAMLVSVTGCARHASGARPASTTLPAGADLADRFAELERRYDARLG

BlaS IYAVDLDSNRTVAHRADDSFAMCSTFKAYLAARILRGAERGELSLDDRVFVDPAALLSNS
BlaF LYAANLQSGRRITHRLDEMFAMCSTFKGYAAARVLQMAEHGEISLDNRVFVDADALVPNS
BlaC VYVPATGTTAAIEYRADERFAFCSTFKAPLVAAVLHQNP--LTHLDKLITYTSDDIRSIS

BlaS PITETHAGGEMTLAELCQAALQRSDNAAANLLLKQIGGP----AEITAFARSIGDQRTRL
BlaF PVTEARAGAEMTLAELCQAALQRSDNTAANLLLKTIGGP----AAVTAFARSVGDERTRL
BlaC PVAQQHVQTGMTIGQLCDAAIRYSDGTAANLLLADLGGPGGGTAAFTGYLRSLGDTVSRL

BlaS DRWETELNSAVPGDPRDTSTPAALAGGFRAVLTGDVLAPPQRQLLDEWMRANETSS--LR
BlaF DRWEVELNSAIPGDPRDTSTAAALAVGYRAILAGDALSPPQRGLLEDWMRANQTSS--MR
BlaC DAEEPELNRDPPGDERDTTTPHAIALVLQQLVLGNALPPDKRALLTDWMARNTTGAKRIR

BlaS AGLPDGWTSADKTGSGDYGSTNDVGIAYGPQGQRILLALMVRTRGDDPNADGFRPLIGEL
BlaF AGLPEGWTTADKTGSGDYGSTNDAGIAFGPDGQRLLLVMMTRSQAHDPKAENLRPLIGEL
BlaC AGFPADWKVIDKTGTGDYGRANDIAVVWSPTGVPYVVAVMSDRAGGGYDAEPREALLAEA

BlaS TALVLPELGVH
BlaF TALVLPSLL--
BlaC ATCVAGVLA--
```

Fig. 1. Multiple alignment of *M. smegmatis* BlaS, *M. fortuitum* BlaF and *M. tuberculosis* H37Rv BlaC β-lactamases using CLUSTAL W version 1.8 (http://clustalw.genome.ad.jp/). The *M. smegmatis* BlaS protein is 70 % and 37 % identical to the *M. fortuitum* BlaF protein and the *M. tuberculosis* BlaC protein, respectively (BLASTP 2.2.4; http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Structural motifs indicative of class A β-lactamases (Ambler, 1980; Ambler *et al.*, 1991) are boxed, while the regions deleted in the *blaC* and *blaS* mutants are underlined.

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```
P99  MMRKS------LCCALLLGISCSALATPVSEKQLAEVVANTITPLMKAQSVPGMAVAVI
K12  -MFKT-------TLCALLITASCSTFAAP--QQINDIVHRTITPLIEQQKIPGMAVAVI
BlaE ----------------------------------------------------MAVAVT
PAO1 MRDTRFPCLCGIAASTLLFATTPAIAGEAPADRLKALVDAAVQPVMKANDIPGLAVAIS

P99  YQGKPHYYTFGKADIAANKPVTPQTLFELGSISKTFTGVLGGDAIARGEISLDDAVTRYW
K12  YQGKPYYFTWGYADIAKKQPVTQQTLFELGSVSKTFTGVLGGDAIARGEIKLSDPTTKYW
BlaE VDGRQHFYEFGVVSKQTQAPVTRDTLFEIGSVSKTFTATLAGYAATRGVLNLDDHPGRYL
PAO1 LKGEPHYFSYGLASKEDGRRVTPETLFEIGSVSKTFTATLAGYALTQDKMRLDDRASQHW

P99  PQLTGKQWQGIRMLDLATYTAGGLPLQVPDEVTDN-ASLLRFYQNWQPQWKPGTTRLYAN
K12  PELTAKQWNGITLLHLATYTAGGLPLQVPDEVKSS-SDLLRFYQNWQPAWAPGTQRLYAN
BlaE PALAGTPIDRAELRNLGTYTAGGLPLQFPESVTDD-EQMIAYFQQFQPVTAPGKIRQYSN
PAO1 PALQGSRFDGISLLDLATYTAGGLPLQFPDSVQKDQAQIRDYYRQWQPTYAPGSQRLYSN

P99  ASIGLFGALAVKPSGMPYEQAMTTRVLKPLKLDHTWINVPKAEEAHYAWGY-RDGKAVRV
K12  SSIGLFGALAVKPSGLSFEQAMQTRVFQPLKLNHTWINVPPAEEKNYAWGY-REGKAVHV
BlaE PSVGLLGHISARALGGQFTDLMQSQILTGLGLRRSFVDVTDEAMDFYAWGYDKKNHPVRV
PAO1 PSIGLFGYLAARSLGQPFERLMEQQVFPALGLEQTHLDVPEAALAQYAQGYGKDDRPLRV

P99  SPGMLDAQAYGVKTNVQDMANWVMANMAPENVADASLKQGIALAQSRYWRIGSMYQGLGW
K12  SPGALDAEAYGVKSTIEDMARWVQSNLKPLDINEKTLQQGIQLAQSRYWQTGDMYQGLGW
BlaE NPGVFDAEAYGVKSTTADMIRFIEHNIDPGAL-EPTLREAVKSTQVGYYKVGPMVQDLGW
PAO1 GPGPLDAEGYGVKTSAADLLRFVDANLHPERL-DRPWAQALDATHRGYYKVGDMTQGLGW

P99  EMLNWPVEANTVVEGSDSKVALAPLPVAEVNPPAPPVKASWVHKTGSTGGFGSYVAFIPE
K12  EMLDWPVNPDSIINGSDNKIALAARPVKAITPPTPAVRASWVHKTGATGGFGSYVAFIPE
BlaE EQYPYPVALDQLLAGNSGEMAMSPQAATAIAPPS--VGSALFNKTGSTDGFGAYAAFVPE
PAO1 EAYDWPISLKRLQAGNSTPMALQPHRIARLPAPQALEGQRLLNKTGSTNGFGAYVAFVPG
                                                              PE

P99   KQIGIVMLANTSYPNPARVEAAYHILEALQ---------
K12   KELGIVMLANKNYPNPARVDAAWQILNALQ---------
BlaE  RRIGIVMLANKNFPIPARVTAAHTVLDALDA--------
PAO1  RDLGLVILANRNYPNAERVKIAYAILSGLEQQGKVPLKR
      KNLGIVMLANKSYPNPA
```

Fig. 2. Multiple alignment using CLUSTAL W version 1.8 (http://clustalw.genome.ad.jp/) of the known class C β-lactamases *Enterobacter cloacae* P99 (GenBank accession no. P05364), *Escherichia coli* K-12 AmpC (GenBank accession no. NP_418574) and *Pseudomonas aeruginosa* PAO1 AmpC (GenBank accession no. NP_252799) with the putative β-lactamase from *M. smegmatis* mc^{2}155, BlaE (GenBank accession no. AY442183). Motifs indicative of class C β-lactamases are boxed. The N-terminal sequence of the purified *M. smegmatis* SN_2 cephalosporinase (Basu *et al.*, 1997) used to identify the putative *M. smegmatis* mc^{2}155 β-lactamase, BlaE, is shown in italics with the homologous region in BlaE in bold. The sequence deleted in the Δ*blaE1* mutant PM976 is underlined.

for the non-β-lactam antibiotics isoniazid and rifampicin (data not shown).

Similarly, wild-type *M. tuberculosis* H37Rv was not susceptible to the β-lactam antibiotics tested, except for ceftriaxone, imipenem, and the amoxicillin/clavulanic acid combination (Table 4). The *blaC* knockout, PM638, showed susceptibility to all β-lactam antibiotics except oxacillin and cefixime. However, there was no increase in the susceptibility of the mutant to ceftriaxone and the susceptibility to imipenem was inconsistent. There was an increase in zone size for amoxicillin/clavulanic acid for PM638, but this was close to the variation for this method, and no such increase was seen for the mutant bearing the vector, control strain PM670 (Table 4). There was no increase in the susceptibility of the mutant to the non-β-lactam antibiotics isoniazid and rifampicin (data not shown).

We determined MICs to further define the extent of susceptibility of PM759 and PM638. As shown in Table 5, the *M. smegmatis* mutant PM759 showed little or no change in MIC values for cefoxitin or ceftriaxone. However, the mutant showed a 16- to 64-fold increase in susceptibility to amoxicillin, ampicillin, carbenicillin and oxacillin. The presence of clavulanic acid resulted in an eight to 16-fold increase in the MIC value for amoxicillin for the wild-type strain and an four- to eightfold increase for the mutant strain.

Wild-type *M. tuberculosis* H37Rv showed a relatively high level of resistance to all β-lactams tested using MIC determination. The inclusion of clavulanic acid reduced the MIC for amoxicillin by at least 16-fold for the wild-type H37Rv and by eightfold for the Δ*blaC1* mutant PM638 The mutant showed a 16-fold increase in susceptibility to

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Table 3. Susceptibility determined by disc diffusion for *M. smegmatis* strains

Zone diameters reported are the mean of triplicate determinations with variation <2 mm. The genotypes of the respective strains are as follows: PM274, *blaS*$^+$; PM759, Δ*blaS1*; PM791, PM759 *attB*::pMP283; PM876, PM759 *attB*::pMV361.*hyg*.

Antibiotic (amount per disc)	Zone diameter (mm) for strain:			
	PM274	PM759	PM791	PM876
Oxacillin (10 μg)*	0	0	0	0
Ampicillin (10 μg)	0	17	0	17
Ampicillin (100 μg)*	0	43	0	43
Piperacillin (100 μg)	0	14	0	11
Mezlocillin (75 μg)	0	12	0	10
Carbenicillin (100 μg)	0	13	0	14
Amoxicillin (20 μg)	0	24	0	22
Amox/clav (20 μg/10 μg)	22	27	21	23
Cefoxitin (30 μg)	0	11	0	11
Cefoxitin (100 μg)*	22	20	22	23
Ceftriaxone (30 μg)	0	0	0	0
Cefixime (5 μg)	0	0	0	0
Imipenem (10 μg)	23	25	27	26

*Antibiotic spotted on blank disc from stock solution, as described in Methods.

amoxicillin and carbenicillin, a 32-fold increase in sensitivity to cefoxitin, and an eightfold increase in sensitivity to ceftriaxone. This differed from the *M. smegmatis* mutant, which did not show an increase in susceptibility to either cefoxitin or ceftriaxone. No changes in the MICs were observed in the *M. tuberculosis* mutant compared to wild-type when the standard anti-tubercular drugs streptomycin, isoniazid, pyrazinamide, ethambutol and rifampicin were tested (data not shown).

Table 4. Susceptibility determined by disc diffusion for *M. tuberculosis* strains

Zone diameters reported are the mean of triplicate determinations with variation <10 mm. The genotypes of the respective strains are as follows: H37Rv, *blaC*$^+$; PM638, Δ*blaC1*; PM669, PM638 *attB*::pMP199; PM670, PM638 *attB*::pMV361.*hyg*.

Antibiotic (amount per disc)	Zone diameter (mm) for strain:			
	H37Rv	PM638	PM669	PM670
Oxacillin (10 μg)*	0	0	0	0
Ampicillin (10 μg)	0	25	0	25
Piperacillin (100 μg)	0	40	0	15
Mezlocillin (75 μg)	0	55	0	30
Carbenicillin (100 μg)	0	60	0	50
Amoxicillin (20 μg)	0	45	0	45
Amox/clav (20 μg/10 μg)	15	60	20	40
Cefoxitin (30 μg)	0	20	0	0
Ceftriaxone (30 μg)	15	20	20	10
Cefixime (5 μg)	0	0	0	0
Imipenem (10 μg)	25	45	25	25

*Antibiotic spotted on blank disc from stock solution, as described in Methods.

Complementation of both the mutant strains PM638 and PM759 with their wild-type β-lactamase genes restored the parental resistance pattern in both disc diffusion tests (Tables 3 and 4) and MIC determinations (Table 5). The *blaC* gene of *M. tuberculosis* was incapable of complementing the *M. smegmatis* Δ*blaS1* mutant, PM759, in single copy but it restored the parental phenotype in multi-copy (disc diffusion data not shown).

Susceptibility studies were also performed on the *M. smegmatis* double β-lactamase knockout, PM976. Disc

Table 5. Susceptibility determined by MIC for *M. smegmatis* and *M. tuberculosis*

The genotypes of *M. smegmatis* strains are as follows: PM274, *blaS*$^+$; PM759, Δ*blaS1*; PM791, PM759 *attB*::pMP283; PM876, PM759 *attB*::pMV361.*hyg*. The genotypes of *M. tuberculosis* strains are as follows: H37Rv, *blaC*$^+$; PM638, Δ*blaC1*; PM669, PM638 *attB*::pMP199; PM670, PM638 *attB*::pMV361.*hyg*. Values are reported in μg ml^{-1}. All MICs were performed in duplicate on at least two independent cultures. Values are reported as a range, or as a single number in cases of non-variant results. ND, Not determined.

Antibiotic	Susceptibility for *M. smegmatis* strains:				Susceptibility for *M. tuberculosis* strains:			
	PM274	PM759	PM791	PM876	H37Rv	PM638	PM669	PM670
Amoxicillin	8–16	0·5–1	32	0·5–1	>256	16	>256	16
Ampicillin	128	2	256	2	ND	ND	ND	ND
Carbenicillin	512	8	>512	8	>960	60	>960	60
Oxacillin	32	>2	32-64	>2	ND	ND	ND	ND
Amoxicillin/clavulanic acid*	1/0·5	0·125/0·0625	1/0·5	0·125/0·0625	16/8	2/1	16/8	2/1
Cefoxitin	2–4	2–4	4–8	4	960	30	960	30
Ceftriaxone	32	16	32–64	8–16	480	60	480	60

*The ratio of amoxicillin : clavulanic acid was maintained at 2 : 1 for all dilutions.

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Table 6. Susceptibility determined by disc diffusion for the *M. smegmatis* double mutant

The genotypes of respective strains are as follows: PM759, Δ*blaS1*; PM976, Δ*blaS1* Δ*blaE1*::*res*. Zone diameters reported are one of triplicate determinations with variation <2 mm.

Antibiotic (amount per disc)	Zone diameter (mm) for strain:	
	PM759	PM976
Oxacillin (10 μg)*	0	0
Ampicillin (10 μg)	20	24
Carbenicillin (100 μg)	16	23
Piperacillin (100 μg)	0	14
Mezlocillin (75 μg)	12	15
Amoxicillin (20 μg)	20	20
Amoxicillin/clavulanate (20/10 μg)	21	19
Cephalothin (30 μg)	0	16
Cefoxitin (30 μg)	11	11
Ceftazidime (30 μg)	0	0
Cefoperazone (75 μg)	11	14
Cefotaxime (30 μg)	0	0
Cefazolin (30 μg)	0	10
Ceftriaxone (30 μg)	0	10
Cefixime (30 μg)	0	0

*Antibiotic spotted on blank disc from stock solution, as described in Methods.

diffusion tests showed little difference between the single β-lactamase knockout, PM759, and the double β-lactamase knockout, PM976 (Table 6). The presence of clavulanic acid did not change the susceptibility of the double mutant to amoxicillin (Table 6). Because the sequence comparisons suggested that BlaE might be a cephalosporinase, a wider variety of cephalosporins was included in the disc diffusion studies. As seen in Table 6, no significant differences in susceptibility to penicillin-based β-lactams were observed, with the exception of piperacillin. Similarly, most of the cephalosporin-based β-lactam antibiotics showed no major differences. However, cephalothin, cefazolin and ceftriaxone showed very small but consistent zones of inhibition with PM976, whereas no zone was observed for PM759. The MICs were determined to be nearly identical for the two strains, PM759 and PM976 (Table 7).

Nitrocefin assays

We performed nitrocefin hydrolysis assays to compare the β-lactamase activity in whole-cell lysates of the wild-type and mutant strains. We chose to use whole-cell lysates since the majority of β-lactamase activity appears to be cell-associated in late-exponential-phase cultures of mycobacteria (data not shown; Fattorini *et al.*, 1991; Zhang *et al.*, 1992). The β-lactamase activity in *M. smegmatis* PM274 and in the single β-lactamase knockout, PM759, lysate is shown in Table 8. A significant decrease in activity was observed in

Table 7. Susceptibility as determined by MIC for the *M. smegmatis* double mutant

The genotypes of respective strains are as follows: PM759, Δ*blaS1*; PM976, Δ*blaS1* Δ*blaE1*::*res*. MIC values are reported in μg ml^{-1}. All MICs were performed in duplicate on at least two independent cultures. Values are reported as a range, or a single number in cases of non-variant results. The ratio of amoxicillin : clavulanic acid (Amox/clav) was maintained at 2 : 1 for all dilutions.

Antibiotic	MIC (μg ml^{-1}) for strain:	
	PM759	PM976
Ampicillin	4	2–4
Carbenicillin	8	4–8
Amoxicillin	0·125–0·25	0·125–0·25
Amox/clav	0·125–0·25/0·0625–0·125	0·125/0·0625
Cefoxitin	4	8
Ceftriaxone	16	16
Cephalothin	2	1

PM759, compared to PM274, with a residual β-lactamase activity present in PM759. The *M. tuberculosis* mutant PM638 also showed significantly reduced β-lactamase activity compared to wild-type, but, in contrast to the *M. smegmatis* mutant, had no detectable residual β-lactamase activity (Table 8). The amount of nitrocefin hydrolysis in the PM638 lysates was essentially the same as that of a negative buffer control. We also noted that the β-lactamase activity in the wild-type strain of *M. tuberculosis* was 10-fold lower than that in wild-type *M. smegmatis*.

A group 2e cephalosporinase has been reported in *M. smegmatis* SN_2 (Basu *et al.*, 1997), suggesting that a similar β-lactamase may be present in *M. smegmatis* mc^2155 that might account for the phenotype of the Δ*blaS1* mutant. We attempted to partially characterize the residual β-lactamase in PM759 through competition assays using benzylpenicillin and the cephalosporin cephalothin. As shown in Fig. 3, the

Table 8. Nitrocefin assays on whole-cell lysates of *M. smegmatis* and *M. tuberculosis* wild-type and β-lactamase knockout strains

Nitrocefin activity is expressed as μg nitrocefin hydrolysed min^{-1} (mg total protein)$^{-1}$ ± SD. Each assay was performed in triplicate.

Strain	Description	Nitrocefin activity
M. smegmatis		
PM274	*blaS*$^+$	31·67 ± 0·92
PM759	Δ*blaS1*	0·6963 ± 0·0177*
PM976	Δ*blaS1* Δ*blaE1*::*res*	0·0172 ± 0·007*
M. tuberculosis		
H37Rv	*blaC*$^+$	2·880 ± 0·111
PM638	Δ*blaC1*	0·0359 ± 0·0018*

*$P<0·01$.

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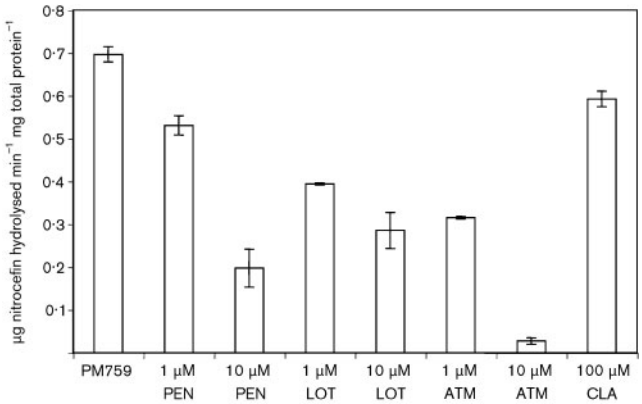

Fig. 3. Nitrocefin competition assays with *M. smegmatis* PM759 whole-cell lysates. All assays were performed in triplicate and on lysates from two cultures. Each column represents the mean of three assays from one lysate and error bars represent the standard deviation. CLA, clavulanic acid; ATM, aztreonam; LOT, cephalothin; PEN, penicillin.

residual β-lactamase activity in PM759 was able to hydrolyse both antibiotics. We found that 10 μM of either benzylpenicillin or cephalothin decreased the nitrocefin hydrolysing activity by 71 % and 59 %, respectively ($P<0{\cdot}001$). Even at the lowest concentration of competitor (1 μM), we found a significant decrease in nitrocefin hydrolysis, 24 % and 43 % for benzylpenicillin and cephalothin, respectively ($P<0{\cdot}002$), suggesting that the activity has an affinity for both classes of β-lactams.

Cephalosporinases are differentiated through their susceptibility to clavulanic acid and aztreonam (Bush *et al.*, 1995). The functional type 1 cephalosporinases are more susceptible to inhibition by aztreonam than by clavulanic acid. Conversely, cephalosporinases of type 2 can be inhibited by low levels of clavulanic acid. Since previous reports suggest the presence of a type 2e cephalosporinase in *M. smegmatis* (Basu *et al.*, 1997), we examined the nitrocefin hydrolysing ability in PM759 in the presence of either clavulanic acid or aztreonam. As shown in Fig. 3, the addition of aztreonam at 1 μM resulted in a 55 % decrease in nitrocefin hydrolysing ability while the addition of 10 μM aztreonam reduced β-lactamase activity to less than 4 % of the level seen in the control reaction ($P<0{\cdot}001$). In contrast, the addition of 100 μM clavulanic acid to the lysate resulted in only a 15 % decrease in β-lactamase activity. While statistically significant ($P<0{\cdot}05$), the effect was much less pronounced than that seen with aztreonam, suggesting that the residual activity in PM759 is a type 1 β-lactamase.

Finally, to demonstrate that this residual β-lactamase activity in PM759 was due to the minor β-lactamase, BlaE, we performed nitrocefin assays on whole-cell lysates of the double mutant PM976. As shown in Table 8, the β-lactamase activity of PM976 was reduced compared to the major β-lactamase mutant, PM759, to a level similar to that seen for the *M. tuberculosis* mutant PM638.

DISCUSSION

The goal of this study was to investigate the contribution of the major β-lactamases, BlaC and BlaS, in *M. tuberculosis* and *M. smegmatis* to β-lactam antibiotic resistance and to examine any additional β-lactamases in these organisms. We have demonstrated that deletion of the major β-lactamases of *M. smegmatis* and *M. tuberculosis* resulted in an increase in susceptibility to β-lactam antibiotics. The *M. smegmatis* β-lactamase-deficient mutant retained a residual β-lactamase activity, and we determined that this activity is the result of an additional β-lactamase, BlaE. However, the contribution of this minor β-lactamase to the resistance of *M. smegmatis* appears to be minimal.

In this study, we showed that the *M. tuberculosis* H37Rv Δ*blaC1* mutant was devoid of any detectable nitrocefin hydrolysing activity. This is in contrast to the results of a previous study suggesting that *M. tuberculosis* H37Ra produces an additional minor β-lactamase with predominant cephalosporinase activity (Voladri *et al.*, 1998). This discrepancy may be due to strain differences, as we have used the strain H37Rv for our work and Voladri *et al.* (1998) used the strain H37Ra. Alternatively, the previous study used purified β-lactamase preparations and consequently may have been more sensitive than our study, which used whole-cell lysates. In addition, the aforementioned study harvested β-lactamase activities from the supernatants of 3–4-week-old cultures. If a minor β-lactamase is produced at a much slower rate and is subsequently secreted or released into the medium due to cell lysis, a 3–4-week-old culture could yield larger amounts of the minor β-lactamase. Our study used whole-cell lysates of younger, late-exponential-phase cultures; the studies in *M. fortuitum* (Fattorini *et al.*, 1991), *M. tuberculosis* (Zhang *et al.*, 1992) and our own data (not shown) indicate that the majority of β-lactamase activity at this stage is cell-associated.

Alternatively, it has been proposed that this minor cephalosporinase of *M. tuberculosis* is not a β-lactamase *per se*, but is D,D-carboxypeptidase, capable of hydrolysing β-lactam antibiotics (Voladri *et al.*, 1998).

BlaS, the major β-lactamase of *M. smegmatis* described here, shows a high degree of homology to the molecular class A β-lactamase enzymes (Ambler, 1980; Ambler *et al.*, 1991). This same classification has been previously suggested based on N-terminal sequencing of a purified β-lactamase from *M. smegmatis* mc^{2}155 (Quinting *et al.*, 1997). The analysis of the coding region for the enzyme reported in this study supports its molecular class A β-lactamase classification. Previous biochemical studies suggested that the *M. smegmatis* major β-lactamase hydrolyses penicillins and cephalosporins in an equally efficient manner. These biochemical data support the class A designation indicated by protein homology.

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This study presents the first description of a minor β-lactamase gene in *M. smegmatis*. BlaE was identified based on an N-terminal sequence reported from a purified cephalosporinase in *M. smegmatis* SN_2 (Basu *et al.*, 1997). However, in that same work, biochemical studies suggested a group 2e functional classification for the enzyme. Our study shows that the protein sequence, activity and inhibitor profile are consistent with the classification of the BlaE enzyme as a group 1 cephalosporinase. However, substrate and inhibitor profiles using purified enzyme are necessary to confirm this classification.

We found a higher β-lactamase activity in extracts of wild-type *M. smegmatis* than in extracts of wild-type *M. tuberculosis*. This might be due to differences in lysate preparation, as we had to pass the *M. tuberculosis* lysates though a 0·2 μm filter for safety purposes. However, control experiments (not shown) indicated that filtration does not reduce β-lactamase detection in the lysates. Alternatively, the reduced β-lactamase activity of *M. tuberculosis* could be the result either of accumulated changes within the coding region of *blaC* or of the weakness of the *blaC* promoter compared to the *blaS* promoter. Our complementation studies suggest that the former is more likely, since the wild-type *M. tuberculosis blaC* gene, when expressed from the strong heterologous *groEL* promoter, is able to restore a wild-type phenotype in the *M. smegmatis* Δ*blaS1* mutant when it is in multi-copy, but not when it is in single copy (data not shown). Furthermore, the amino acid identity between the BlaC and BlaS proteins is only 37 %, making these proteins as similar to each other as they are to β-lactamases of non-mycobacterial species. We hypothesize that the *blaC* gene of *M. tuberculosis* has accumulated mildly deleterious mutations over time that have decreased the activity of the BlaC enzyme. Such mutations would likely be tolerated, as there is no selective pressure on an obligate human pathogen such as *M. tuberculosis* to maintain a functioning β-lactamase enzyme. In contrast, an environmental organism such as *M. smegmatis* would presumably rely on resistance mechanisms such as β-lactamases to ensure its survival and is under selective pressure to maintain a higher level of β-lactamase activity.

Disc diffusion tests showed an overall increase in susceptibility, relative to the wild-type of both mutants, to most β-lactam antibiotics. Specifically, the greatest increase was observed for the penicillin-based β-lactam antibiotics. This was expected for *M. tuberculosis*, as initial biochemical descriptions of BlaC indicated that it possessed a predominant penicillinase activity. We observed a similar susceptibility profile in the BlaS mutant of *M. smegmatis*. However, little or no change in susceptibility was observed for oxacillin, ceftriaxone or cefixime (depending upon the species). Essentially no differences were observed for the *M. smegmatis* double β-lactamase mutant PM976.

MIC determination confirmed the differences in the susceptibility patterns observed between wild-type and mutant strains in the disc diffusion test in both *M. smegmatis* and *M. tuberculosis*. However, some discrepancies were readily apparent with oxacillin, ceftriaxone, cefoxitin and the comparison of amoxicillin and amoxicillin/clavulanic acid. The differences observed could be due to subtle differences between growth on liquid versus solid media, differences in inocula size between MIC and disc diffusion tests, or a combination of these factors.

The *M. tuberculosis* knockout, PM638, appeared to be more susceptible to β-lactam antibiotics, as measured by disc diffusion, than was the *M. smegmatis* mutant PM759. It is difficult to make the same comparison for the MIC values between the two species, due to the use of two different methods for MIC determination. However, the fold change in cephalosporin MICs of the *M. tuberculosis* mutant compared to wild-type was greater than that observed between the wild-type and mutant *M. smegmatis* strains. Differences in cell envelope permeability may be responsible for these observations.

Production of β-lactamases and the low permeability of the mycobacterial cell wall are believed to act synergistically to produce a β-lactam resistance phenotype (Jarlier & Nikaido, 1994). The roles of β-lactamase production and low permeability have been studied in *M. chelonae*. While the cell wall of *M. chelonae* is 10-fold less permeable than that of *Pseudomonas aeruginosa* and 1000-fold less permeable than that of *E. coli*, low permeability by itself is insufficient to produce high resistance to β-lactam antibiotics (Jarlier & Nikaido, 1990). The low permeability of *M. chelonae* acts synergistically with its β-lactamase production to produce an organism with extreme resistance to β-lactam antibiotics (Jarlier *et al.*, 1991). Mycobacterial permeability studies show that fast-growing saprophytic organisms such as *M. chelonae* (Jarlier & Nikaido, 1990) and *M. smegmatis* (Trias & Benz, 1994) are less permeable to β-lactam antibiotics than slow-growing obligate pathogens such as *M. tuberculosis* (Jarlier & Nikaido, 1994). It is reasonable to surmise that higher permeability is responsible for the increase in susceptibility seen with *M. tuberculosis*.

An additional key element in the entry of the hydrophilic β-lactam antibiotics is the presence of porins in the mycobacterial cell wall. Substantial information exists regarding the porins and porin genes of *M. smegmatis*, while less is known regarding the porin(s) of *M. tuberculosis* (Niederweis, 2003). Hydrophilic compounds, such as the β-lactam antibiotics, are predicted to permeate the mycobacterial cell envelope through porins (Trias & Benz, 1994). Specifically, the porins of *M. smegmatis* and *M. chelonae* appear to be cationic or zwitterionic selective. Thus, the rate of permeation by β-lactam antibiotics is most likely dependent upon the overall charge of the molecule. A recent study showed that porins do influence the uptake of antibiotics, particularly β-lactams, in *M. tuberculosis* (Mailaender *et al.*, 2004). In addition, it has also been noted that the *M. smegmatis* porin density is significantly less than that observed in Gram-negative bacteria (Engelhardt *et al.*, 2002). Porin selectivity and density may contribute to the

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differences in susceptibility observed here between *M. smegmatis* and *M. tuberculosis*.

Our results suggest that the major β-lactamases contribute significantly to the resistance of *M. tuberculosis* and *M. smegmatis* to β-lactam antibiotics. Our biochemical evidence indicates that there is only one β-lactamase in *M. tuberculosis* and two in *M. smegmatis*. However, the antibiotic susceptibility data suggest that there may be additional, difficult to detect, β-lactamase enzymes in these organisms. The susceptibility of the mutants (PM638 and PM759) to amoxicillin as assayed by MIC was increased in the presence of clavulanic acid by four- to eightfold in certain cases (Table 5), but not in others (Table 7). In addition, an effect of clavulanic acid on the mutants was not seen for the disc diffusion tests (Table 3 and 4). This could suggest the presence of additional clavulanic acid-sensitive, but low-activity β-lactamases in both strains. We did not detect any additional β-lactamase activity in the mutants PM638 (Δ*blaC1*) and PM976 (Δ*blaS1* Δ*blaE1* : : *res*). There is a possibility that additional β-lactamases in the lysates were lost in the cell-wall fraction if they were somehow tightly associated with the cell wall. Our method to prepare lysates of *M. smegmatis* included a centrifugation step to pellet debris that would also pellet the cell wall; however, the same centrifugation step was done at a much slower speed, insufficient to pellet the cell wall, for the preparation of the *M. tuberculosis* lysates.

Another possibility is that the effect of clavulanic acid on the amoxicillin susceptibility of the mutants is not due to inhibition of β-lactamases but is the result of effects that clavulanic acid can have on cell wall biosynthesis. It has been previously shown that β-lactamase-negative pneumococci grown with subinhibitory concentrations of clavulanic acid are more susceptible to β-lactam antibiotics and have alterations in their cell wall indicative of inhibition of a D,D-carboxypeptidase (Severin *et al.*, 1997). We surmise that a similar phenomenon might occur in mycobacteria growing in the presence of clavulanic acid.

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REFERENCES

Ambler, R. P. (1980). The structure of β-lactamases. *Philos Trans R Soc Lond B Biol Sci* **289**, 321–331.

Ambler, R. P., Coulson, A. F., Frere, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G. & Waley, S. G. (1991). A standard numbering scheme for the class A β-lactamases. *Biochem J* **276**, 269–270.

Basu, D., Narayankumar, D. V., Van Beeumen, J. & Basu, J. (1997). Characterization of a β-lactamase from *Mycobacterium smegmatis* SN_2. *Biochem Mol Biol Int* **43**, 557–562.

Bush, K., Jacoby, G. A. & Medeiros, A. A. (1995). A functional classification scheme for β-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother* **39**, 1211–1233.

Casal, M. J., Rodriguez, F. C., Luna, M. D. & Benavente, M. C. (1987). In vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *Mycobacterium avium*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae* to ticarcillin in combination with clavulanic acid. *Antimicrob Agents Chemother* **31**, 132–133.

Chambers, H. F., Moreau, D., Yajko, D. & 7 other authors (1995). Can penicillins and other β-lactam antibiotics be used to treat tuberculosis? *Antimicrob Agents Chemother* **39**, 2620–2624.

Chambers, H. F., Kocagoz, T., Sipit, T., Turner, J. & Hopewell, P. C. (1998). Activity of amoxicillin/clavulanate in patients with tuberculosis. *Clin Infect Dis* **26**, 874–877.

Cole, S. T., Brosch, R., Parkhill, J. & 39 other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.

Consaul, S. A., Jacobs, W. R., Jr & Pavelka, M. S., Jr (2003). Extragenic suppression of the requirement for diaminopimelate in diaminopimelate auxotrophs of *Mycobacterium smegmatis*. *FEMS Microbiol Lett* **225**, 131–135.

Cynamon, M. H. & Palmer, G. S. (1983). In vitro activity of amoxicillin in combination with clavulanic acid against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **24**, 429–431.

Cynamon, M. H. & Patapow, A. (1981). In vitro susceptibility of *Mycobacterium fortuitum* to cefoxitin. *Antimicrob Agents Chemother* **19**, 205–207.

Dye, C., Scheele, S., Dolin, P., Pathania, V. & Raviglione, M. C. (1999). Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* **282**, 677–686.

Engelhardt, H., Heinz, C. & Niederweis, M. (2002). A tetrameric porin limits the cell wall permeability of *Mycobacterium smegmatis*. *J Biol Chem* **277**, 37567–37572.

Fattorini, L., Scardaci, G., Jin, S. H., Amicosante, G., Franceschini, N., Oratore, A. & Orefici, G. (1991). β-Lactamase of *Mycobacterium fortuitum*: kinetics of production and relationship with resistance to β-lactam antibiotics. *Antimicrob Agents Chemother* **35**, 1760–1764.

Hackbarth, C. J., Unsal, I. & Chambers, H. F. (1997). Cloning and sequence analysis of a class A β-lactamase from *Mycobacterium tuberculosis* H37Ra. *Antimicrob Agents Chemother* **41**, 1182–1185.

Jacobs, W. R., Jr, Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R. A., Jones, W., Barletta, R. G. & Bloom, B. R. (1991). Genetic systems for mycobacteria. *Methods Enzymol* **204**, 537–555.

Jarboe, E., Stone, B. L., Burman, W. J., Wallace, R. J., Jr, Brown, B. A., Reves, R. R. & Wilson, M. L. (1998). Evaluation of a disk diffusion method for determining susceptibility of *Mycobacterium avium* complex to clarithromycin. *Diagn Microbiol Infect Dis* **30**, 197–203.

Jarlier, V. & Nikaido, H. (1990). Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. *J Bacteriol* **172**, 1418–1423.

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Jarlier, V. & Nikaido, H. (1994). Mycobacterial cell wall: structure and role in natural resistance to antibiotics. *FEMS Microbiol Lett* **123**, 11–18.

Jarlier, V., Gutmann, L. & Nikaido, H. (1991). Interplay of cell wall barrier and β-lactamase activity determines high resistance to β-lactam antibiotics in *Mycobacterium chelonae*. *Antimicrob Agents Chemother* **35**, 1937–1939.

Jorgensen, J. H., Turnidge, J. D. & Washington, J. A. (1999). Antibacterial susceptibility tests: dilution and disk diffusion methods. In *Manual of Clinical Microbiology*, pp. 1526–1543. Edited by P. R. Murray, E. J. Baron, M. A. Pfaller, F. C. Tenover & R. H. Yolken. Washington, DC: American Society for Microbiology.

Kaneda, S. & Yabu, K. (1983). Purification and some properties of β-lactamase from *Mycobacterium smegmatis*. *Microbiol Immunol* **27**, 191–193.

Kasik, J. E. (1979). Mycobacterial β-lactamases. In *β-Lactamases*, pp. 339–350. Edited by J. M. T. Hamilton-Miller & J. T. Smith. New York: Academic Press.

Kwon, H. H., Tomioka, H. & Saito, H. (1995). Distribution and characterization of β-lactamases of mycobacteria and related organisms. *Tuber Lung Dis* **76**, 141–148.

Li, X.-Z., Zhang, L. & Nikaido, H. (2004). Efflux-pump mediated intrinsic drug resistance in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* **48**, 2415–2423.

Mailaender, C., Reiling, N., Engelhardt, H., Bossmann, S., Ehlers, S. & Niederweis, M. (2004). The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. *Microbiology* **150**, 853–864.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Nadler, J. P., Berger, J., Nord, J. A., Cofsky, R. & Saxena, M. (1991). Amoxicillin-clavulanic acid for treating drug-resistant *Mycobacterium tuberculosis*. *Chest* **99**, 1025–1026.

Niederweis, M. (2003). Mycobacterial porins – new channel proteins in unique outer membranes. *Mol Microbiol* **49**, 1167–1177.

O'Callaghan, C. H., Morris, A., Kirby, S. M. & Shingler, A. H. (1972). Novel method for detection of β-lactamases by using a chromogenic cephalosporin substrate. *Antimicrob Agents Chemother* **1**, 283–288.

Pavelka, M. S., Jr & Jacobs, W. R., Jr (1996). Biosynthesis of diaminopimelate, the precursor of lysine and a component of peptidoglycan, is an essential function of *Mycobacterium smegmatis*. *J Bacteriol* **178**, 6496–6507.

Pavelka, M. S., Jr & Jacobs, W. R., Jr (1999). Comparison of the construction of unmarked deletion mutations in *Mycobacterium smegmatis*, *Mycobacterium bovis* bacillus Calmette-Guerin, and *Mycobacterium tuberculosis* H37Rv by allelic exchange. *J Bacteriol* **181**, 4780–4789.

Quinting, B., Galleni, M., Timm, J., Gicquel, B., Amicosante, G. & Frere, J. M. (1997). Purification and properties of the *Mycobacterium smegmatis* mc(2)155 β-lactamase. *FEMS Microbiol Lett* **149**, 11–15.

Segura, C., Salvado, M., Collado, I., Chaves, J. & Coira, A. (1998). Contribution of β-lactamases to β-lactam susceptibilities of susceptible and multidrug-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob Agents Chemother* **42**, 1524–1526.

Severin, A., Severina, E. & Tomasz, A. (1997). Abnormal physiological properties and altered cell wall composition in *Streptococcus pneumoniae* grown in the presence of clavulanic acid. *Antimicrob Agents Chemother* **41**, 504–510.

Sorg, T. B. & Cynamon, M. H. (1987). Comparison of four β-lactamase inhibitors in combination with ampicillin against *Mycobacterium tuberculosis*. *J Antimicrob Chemother* **19**, 59–64.

Trias, J. & Benz, R. (1994). Permeability of the cell wall of *Mycobacterium smegmatis*. *Mol Microbiol* **14**, 283–290.

Utrup, L. J., Moore, T. D., Actor, P. & Poupard, J. A. (1995). Susceptibilities of nontuberculosis mycobacterial species to amoxicillin-clavulanic acid alone and in combination with antimycobacterial agents. *Antimicrob Agents Chemother* **39**, 1454–1457.

Voladri, R. K., Lakey, D. L., Hennigan, S. H., Menzies, B. E., Edwards, K. M. & Kernodle, D. S. (1998). Recombinant expression and characterization of the major β-lactamase of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **42**, 1375–1381.

Wallace, R. J., Jr, Dalovisio, J. R. & Pankey, G. A. (1979). Disk diffusion testing of susceptibility of *Mycobacterium fortuitum* and *Mycobacterium chelonei* to antibacterial agents. *Antimicrob Agents Chemother* **16**, 611–614.

Wong, C. S., Palmer, G. S. & Cynamon, M. H. (1988). In-vitro susceptibility of *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium kansasii* to amoxycillin and ticarcillin in combination with clavulanic acid. *J Antimicrob Chemother* **22**, 863–866.

Yabu, K., Kaneda, S. & Ochiai, T. (1985). Relationship between β-lactamase activity and resistance to β-lactam antibiotics in *Mycobacterium smegmatis*. *Microbiol Immunol* **29**, 803–809.

Zhang, Y., Steingrube, V. A. & Wallace, R. J., Jr (1992). Beta-lactamase inhibitors and the inducibility of the beta-lactamases of *Mycobacterium tuberculosis*. *Am Rev Respir Dis* **145**, 657–660.

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