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Transcription and expression analysis, using *lacZ* and *phoA* gene fusions, of *Mycobacterium fortuitum* β-lactamase genes cloned from a natural isolate and a high-level β-lactamase producer

J. Timm,[1]* M. G. Perilli,[2] C. Duez,[3] J. Trias,[1] G. Orefici,[4] L. Fattorini,[4] G. Amicosante,[2] A. Oratore,[2] B. Joris,[3] J. M. Frère,[3] A. P. Pugsley[5] and B. Gicquel[1]

[1]*Unité de Génétique Mycobactérienne, CNRS URA 1300, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris, Cedex 15, France.*

[2]*Department of Biomedical Sciences, Technology and Biometry, University of L'Aquila, Localitá Collemaggio, L'Aquila, Italy.*

[3]*Laboratoire d'Enzymologie et Centre d'Ingéniérie de Protéines, Université de Liège, Institut de Chimie, B-4000 Sart Tilman, Liège, Belgium.*

[4]*Laboratorio de Batteriologia e Micologia Medica, Istituto Superiore di Sanità, Roma, Italy.*

[5]*Unité de Génétique Moléculaire, CNRS URA 1149, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris, Cedex 15, France.*

Summary

The gene encoding a class A β-lactamase was cloned from a natural isolate of *Mycobacterium fortuitum* (*blaF*) and from a high-level amoxicillin-resistant mutant that produces large amounts of β-lactamase (*blaF). The nucleotide sequences of the two genes differ at 11 positions, including two in the region upstream from the coding sequence. Gene fusions to *Escherichia coli lacZ* and transcription and expression analysis of the cloned genes in *Mycobacterium smegmatis* indicated that high-level production of the β-lactamase in the mutant is mainly or wholly due to a single base pair difference in the promoter. These analyses also showed that transcription and translation start at the same position. A comparison of the amino acid sequence of BlaF, as predicted from the nucleotide sequence, with the determined *N*-terminal amino acid sequence indicated the presence of a typical signal peptide. The fusion of *blaF* (or *blaF**) to the *E. coli* gene *phoA* resulted in the production of BlaF–PhoA hybrid proteins that had alkaline phosphatase activity. These results demonstrate that *phoA* can be used as a reporter gene for studying protein export in mycobacteria.**

Received 29 October, 1993; revised 8 February, 1994; accepted 10 February, 1994. *For correspondence. Tel. (1) 45688840; Fax (1) 45688843; e-mail: jtimm@pasteur.fr.

Introduction

The genus *Mycobacterium*, a highly heterogeneous group of organisms, can be divided into slow-growing and fast-growing species. The first group comprises pathogens of man or other animals, and includes *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Fast-growing mycobacteria are saprophytes, like *M. smegmatis*, although some species, e.g. *M. fortuitum*, have also been implicated in human disease (Wallace *et al.*, 1983). A characteristic feature of most mycobacteria is their natural high-level resistance to a large number of antibiotics. This is believed to be due to the low permeability of the mycobacterial cell envelope (Nikaido and Jarlier, 1991). In addition to this, other factors, such as antibiotic-modifying enzymes, may be implicated in high-resistance phenotypes observed in both clinical and natural isolates.

Among *M. tuberculosis* strains, high-level antibiotic resistance was shown to be acquired during or after antibiotherapy (Kochi *et al.*, 1993). These drug-resistant strains are supposed to carry mutations affecting the drug targets. Recent observations supporting this idea are provided by analyses of rifampicin resistance in *M. leprae* (Honoré and Cole, 1993) and *M. tuberculosis* (Telenti *et al.*, 1993), and of isoniazid resistance in *M. tuberculosis* (Zhang *et al.*, 1992), which are caused by mutations in RNA polymerase genes and in the catalase-peroxidase gene, respectively. Except for β-lactamases, antibiotic-modifying activity has not been identified so far in *M. tuberculosis*, but aminoglycoside-modifying activities, particularly aminoglycoside-acetyltransferases (Udou *et al.*, 1987) and phosphotransferases (Hull *et al.*, 1984; our unpublished results), have been detected in extracts of fast-growing mycobacteria. However, it was not possible to demonstrate a positive correlation between resistance levels and the degree of aminoglycoside inactivation in these bacteria (Udou *et al.*, 1989).

Plasmids and transposons are often responsible for the dissemination of antibiotic-resistance genes among

bacteria (Mazodier and Davies, 1991). Plasmids have been found in various species of fast-growing mycobacteria as well as in organisms of the *Mycobacterium avium–intracellulare–scrofulaceum* complex (Martín *et al.*, 1990a; Crawford and Falkinham III, 1990). They have been shown to confer resistance to copper, mercury and cadmium in *Mycobacterium scrofulaceum*, and to carry DNA restriction–modification determinants in *M. avium*. Until now, mycobacterial plasmids have not been associated with resistance to clinically used antibiotics, but a transposon, Tn*610*, carrying a sulphonamide-resistance gene nearly identical to that found in the Tn*21* family of transposons, was identified in *Mycobacterium fortuitum* (Martín *et al.*, 1990b). These observations strongly suggest that horizontal transfer of antibiotic-resistance genes could occur in mycobacteria.

Beta-lactamase has been detected in most mycobacterial species, including *M. tuberculosis*, *M. bovis*, *M. kansasii*, *M. smegmatis*, *M. fortuitum*, *M. phlei*, *M. avium-intracellulare* and *M. lacticola*, but not in *M. marinum*, or *M. xenopi* (Finch, 1986; Kasik, 1979; Stormer and Falkinham III, 1989). Its role in the resistance of *M. fortuitum* to β-lactams has been recently clarified by Fattorini and co-workers. Using a series of mutants of *M. fortuitum* exhibiting modulated β-lactamase production, they showed a positive correlation between β-lactam resistance and β-lactamase activity. The *M. fortuitum* enzyme was purified and shown to be a class A secreted β-lactamase (Amicosante *et al.*, 1990; Fattorini *et al.*, 1991).

In the present study, the isolation and characterization of the *M. fortuitum* β-lactamase gene were undertaken to elucidate the phylogeny, molecular properties, gene regulation, and secretory aspects of the protein. The gene was cloned from a *M. fortuitum* mutant that produced unusually high amounts of β-lactamase (Fattorini *et al.*, 1989) as well as from a natural isolate. The nucleotide sequences of the gene from the mutant, *blaF**, and from the natural isolate, *blaF*, were determined. Expression of these genes in *M. smegmatis* was found to confer high-level resistance to the β-lactam ampicillin. To clarify the role of the sequence differences in the higher level of resistance conferred by *blaF**, gene fusion experiments, using *lacZ* and *phoA* as reporter genes, and transcriptional analysis were carried out in *M. smegmatis* expressing the different *blaF* alleles. Our results indicate that high-level production of the β-lactamase in the mutant is mainly or wholly due to a single base pair difference in the promoter.

Results

Cloning and sequencing of β-lactamase genes from M. fortuitum

The gene encoding the *M. fortuitum* β-lactamase was cloned from one of the overproducing mutants obtained by Fattorini *et al.* (1989), strain D316, and from a natural isolate, FC1 (Zaragoza collection), using a combination of oligonucleotide probing of genomic DNA libraries and *in vitro* synthesis by polymerase chain reaction (PCR) amplification (see the *Experimental procedures*).

The nucleotide sequences of the gene from the mutant, *blaF**, and from the natural isolate, *blaF*, as well as their flanking regions (Fig. 1 and data not shown) were determined. They are identical except for 11 base pair substitutions. They carry an open reading frame (ORF) that extends from a putative ATG start codon at position 1275 to a TGA at position 2157, encoding a polypeptide of 294 amino acids. The *N*-terminal 32 amino acids of this polypeptide resemble a typical amino-terminal signal peptide, with a charged domain (Arg-6, Arg-7), followed by a hydrophobic core, turn-inducing amino acids (Gly-25, Gly-26, Pro-29), and an 'Ala-X-Ala' box (Ala-30, Ala-32) that could be a typical signal peptidase cleavage site (Pugsley, 1993). This segment is followed by an amino acid sequence identical to that reported for the *N*-terminal region of mature *M. fortuitum* β-lactamase (Amicosante *et al.*, 1990). Further computer searches in the GenBank data base revealed homology between the primary structure of this polypeptide and those of other class A β-lactamases (Fig. 2). Notably, all the structural elements characteristic of β-lactamases whose sequences are known (Joris *et al.*, 1991) are present in BlaF.

Promoter analysis

Among the 11 sequence differences identified between the *blaF* and *blaF** fragments, two are located upstream of the putative start codon, at positions 948 and 1265 (Fig. 1). To investigate whether these sequence differences had an effect on transcription, RNA from *M. smegmatis* (pIPJ47) and from *M. smegmatis* (pIPJ47*) was subjected to Northern analysis using a 3′ extremity PCR fragment of *blaF* as a probe. pIPJ47* is an *Escherichia coli*–mycobacteria shuttle plasmid that contains a hybrid gene composed of the putative regulatory sequences and *N*-terminal coding segment of *blaF** fused to the *C*-terminal encoding segment of *blaF* (see the *Experimental procedures*). pIPJ47 contains sequences derived uniquely from *blaF*. Thus these plasmids differ only at two nucleotides within the coding sequence, in addition to those located upstream of the putative initiation codon. As a negative control, we used RNA from *M. smegmatis* harbouring the *E. coli*–mycobacteria shuttle plasmid pRR3 (Ranes *et al.*, 1990). These experiments showed that the level of transcription of *blaF** was considerably higher than that of *blaF*. We therefore used high-resolution S1 mapping to show that transcription of both genes starts at the same site (Fig. 3). These experiments revealed that the 5′-end of both mRNAs corresponds to the first base of the

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TGCTCGGCGG  ACTCCGGGTG  GTCACTGAGT  ACATCCCGTC  GGTGCGTTCG  GCATCGGTCG        60
GGGTGTGGGT  GGGTGTCGGC  TCCCGCGACG  AAGGACGAAG  CGTCGCGGGT  GCCGCCCACT       120
TCTTGGAGCA  TCTGCTGTTC  AAGGCCACCC  CGACGCGCAC  GCGGTCGACA  TCGCGCAGGC       180
TGTCGATGCC  GTCGGCGGTG  AGCTGAACGC  GTTCACCACG  CGCGAGCACA  CCTGTTACTA       240
CGCGCATGTG  CTCGACTCCG  ACCTGGAGCT  CGCGGTCGAC  CTGGTGCGCC  GATGTCGTGT       300
TGCGTGGGCG  TTGTGCCACC  GAGGATGTCG  AAGTGGAGCG  CGACGTCGTC  CTCGAGGAGA       360
TCGCCATGCG  TGACGACGAT  CCCGAGGACA  GCCTCGGCGA  CGTGTTCCTC  TCGGCGATGT       420
TCGGCGATCA  CCCGGTGGGA  CGTCCGGTGA  TCGGCAGCGT  CGAGTCGATC  GAGACCATGA       480
CGCGTGCACA  GCTGCATTCG  TTCCACGTCC  GGCGTTACAC  ACCCGAACGG  ATGATCGTGG       540
CGGTGGCCGG  CAACGTCGAC  CACGACGTGT  GGTGTCGTTG  GTCCGAGAGC  ATTTCGGCCC       600
CCGGCTGGAG  GCCGACGTTC  CGCGGTGGCT  CCCCGTAAGG  CTCGGGACGG  GTCGGTGGTA       660
AGCCATCGCT  GCTCGTGGTC  GACCGCGACG  GGGAACAGTC  CCATGTCTCG  CTGGGCGTTC       720
GCACGCCCGG  CCGGCACTGG  GAGCACCGGT  GGGCCCTGTC  GGTGTTGAAC  ACCGCGCTGG       780
GAGGCGGGCT  CAGTTCTCGT  CTGTTCCAAC  AGATTCGCGA  GTCCCGCGGC  CTGGCCTACC       840
TCGGTGTACT  CGACCGTGGA  CCACTTCGCG  ACAGCGGGGC  TCTGTCGGTG  TATGCGGGAT       900
GTCAGCCGGA  ACGTTTCGAC  GAAGTGGTGC  GGGTGACCAC  CGAAGTTCTG  GAAGGTGTTG       960
                                                          T
CCAGAGACGG  GATCACCGCC  GACGAATGCC  GGATCGCCAA  AGGCTCGTTG  CGCGGTGGGC      1020
TGGTGCTCGG  CCTGGAGGAT  TCCGGATCAC  GTATGCACCG  GATCGGCCGT  AGCGAGCTCA      1080
                                                                XbaI
ATTACGGTGA  GCACCGGACC  ATCGACCACA  CGCTGGCCCA  GATCGAGGCA  GTCACTCTAG      1140
AAGAGGTCAA  CGCCGTCGCT  CACCAGTTGC  GTGTCGCGGG  ACTACGGTGC  CGCCGTACTC      1200
GGTCCCTATA  GTTCGAAAAA  GGCGCTGCAC  AACAGCTTCA  AACTATCGCC  GGCTGACCCG      1260
CTACGCTGGG  TCCA   ATG  ACC  GGA  CTA  TCG  CGA  CGC  AAC  GTT  CTG  ATC      1307
    A              Met  Thr  Gly  Leu  Ser  Arg  Arg  Asn  Val  Leu  Ile

GGT  TCG  CTC  GTG  GCG  GCA  GCT  GCC  GTC  GGC  GCC  GGC  GTC  GGT  GGC      1352
Gly  Ser  Leu  Val  Ala  Ala  Ala  Ala  Val  Gly  Ala  Gly  Val  Gly  Gly

GCC  GCA  CCG  GCA  TTC  GCG  GCA  CCG  ATC  GAT  GAC  CAG  CTG  GCG  GAA      1397
Ala  Ala  Pro  Ala  Phe  Ala  Ala  Pro  Ile  Asp  Asp  Gln  Leu  Ala  Glu
                          ↑
CTG  GAG  CGT  CGG  GAC  AAC  GTC  CTG  ATC  GGC  TTG  TAC  GCA  GCC  AAT      1442
Leu  Glu  Arg  Arg  Asp  Asn  Val  Leu  Ile  Gly  Leu  Tyr  Ala  Ala  Asn

CTG  CAG  TCT  GGG  CGG  AGG  ATC  ACG  CAC  CGT  CTC  GAC  GAG  ATG  TTC      1487
Leu  Gln  Ser  Gly  Arg  Arg  Ile  Thr  His  Arg   C   Asp  Glu  Met  Phe
                                                 Leu
                                                 Pro

GCG  ATG  TGC  TCG  ACG  TTC  AAG  GGC  TAC  GCC  GCT  GCG  CGG  GTG  CTG      1532
Ala  Met  Cys  Ser  Thr  Phe  Lys  Gly  Tyr   T   Ala  Ala  Arg  Val  Leu
                                             Ala
                                             Val

                                  BglII
CAG  ATG  GCC  GAG  CAC  GGC  GAG  ATC  TCA                               1559
Gln  Met  Ala  Glu  His  Gly  Glu  Ile  Ser
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Fig. 1. Nucleotide sequences of 5′ extremities of *blaF* and *blaF** and their flanking regions. The nucleotide sequences of *blaF* and its flankling regions were determined from pIPJ39. *blaF** and its flanking regions were sequenced from pBF1 and pIPJ71. The deduced amino acid sequence of BlaF is given below the DNA sequence. The base pair substitutions found in *blaF**, and the corresponding amino acid changes, are shown in italics. The amino acid sequence of the *N*-terminus of BlaF, determined by Amicosante *et al.* (1990), is in bold characters. The complete sequence data, including the sequence 3′ to those shown here, appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Libraries under the accession number L25634.

presumed translation initiation codon (position 1275). Moreover, the intensity of the signal observed with the same amount of total RNA was approximately 10-fold higher for the *blaF** product, as measured using a PDQUEST gel analyser.

To demonstrate that only the mismatch located at position 1265 had an effect on promoter activity, we created gene fusions using a truncated *lacZ* gene as a reporter (Fig. 4). Fragments containing the upstream region and the presumed start codon from *blaF* or from *blaF** were synthesized *in vitro* by PCR amplification and cloned into the promoter-probe vector pJEM13 (our unpublished data), resulting in plasmids pIPJ66 and pIPJ66*. By replacing a *Bam*HI–*Xba*I fragment in pIPJ66 with that from pIPJ66*, we generated a new plasmid (pIPJ75) that differed from pIPJ66 only at position 1265. Sonicated extracts of *M. smegmatis* carrying pIPJ66*, pIPJ66 or pIPJ75, contained 897, 135, and 158 units of

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                    40          50          60          70          80
                                                        *
S. fr.      EGRLRALERT HDARLGAFAY DTGTGRTVAY RADERFPIAS MFKTIAVAAV
S. la.      LRQLRALEQE HSARLGVYAR DTATGRTVLH RAEERFPMCS VFKTLAVAAV
S. al.      ERRLAGLERA SGARLGVYAY DTGSGRTVAY RADELFPMCS VFKTLSSAAV
B. li.      KDDFAKLEEQ FDAKLGIFAL DTGTNRTVAY RPDERFAFAS TIKALTVGVL
M. fo.      DDQLAELERR DNVLIGLYAA NLQSGRRITH RLDEMFAMCS TFKGYAAARV
Consensus   e..laaLEr. hdarlGvyAy dtgtgRtvay RadErFpmcS .fKtlavaav

                90          100                     110         120
S. fr.      LRDLDRDGEV LARRVHYTAD ...YVKRSGY SPVTGLPENV A.NGMTVAEL
S. la.      LRDLDRDGEF LATRLFYTEQ ...EVKDSGF GPVTGLPENL A.AGMTVERL
S. al.      LRDLDRNGEF LSRRILYTQD D.VEQAD.GR APETGKPQNL ANAQLTVEEL
B. li.      LQQ..KSIED LNQRITYTRD DLV.....NY NPIT...EKH VDTGMTLKEL
M. fo.      LQMAEHGEIS LDNRVFVDAD ALV.....PN SPVT...EAR AGAEMTLAEL
Consensus   Lrdldrdgef LarR.fytad ..v.....gy sPvTg.pen. a.agmTv.eL

            130         140         150         160         170
S. fr.      CEATLTRSDN TAANLLLRDL GGPTAVTRFC RSVGDHVTRL DRWEPELNSA
S. la.      CAAAICQSDN AAANLLLREL GGPEAVTRFC RSVGDRTTRL DRWEPELNSA
S. al.      CEVSITASDN CAANLMLREL GGPAAVTRFV RSLGDRVTRL DRWEPELNSA
B. li.      ADASLRYSDN AAQNLILKQI GGPESLKKEL RKIGDEVTNP ERFEPELNEV
M. fo.      CQAALQRSDN TAANLLLKTI GGPAAVTAFA RSVGDERTRL DRWEVELNSA
Consensus   cea.ltrSDN .AaNLlLrel GGP.avtrfc RsvGD.vTrl dRwEpELNsa

            180         190         200         210         220
S. fr.      EPGRVTDTTS PRAIGRTYGR LILGDLLAAH DRERLTRWML DNRTSDERFR
S. la.      EPGRLTDTTP PRAIGATYGE LVLGDALAPR DRERLTGWLL ANTTSTERFR
S. al.      EPGRVTDTTS PRAITRTYGR LVLGDALNPR DRRLLTSWLL ANTTSGDRFR
B. li.      NPGETQDTST ARALVTSLRA FALEDKLPSE KRELLIDWMK RNTTGDALIR
M. fo.      IPGDPRDTST AAALAVGYRA ILAGDALSPP QRGLLEDWMR ANQTSS..MR
Consensus   ePGrvtDTtt prAigrtyg. lvlgDaLapr dRelLtdWml aNtTsderfR

            230         240         250         260         270
S. fr.      KGLPADWLLA DKTGGGDYGT NNDAGVAWPP GRPPVVLAVQ TTRFTPDAEA
S. la.      KGLPADWTLG DKTGGGAYGT NNDAGVTWPP HRPPVVMVVL TTHDRPDAVA
S. al.      AGLPDDWTLG DKTGAGRYGT NNDAGVTWPP GRAPIVLTVL TAKTEQDAAR
B. li.      AGVPDGWEVA DKTGAASYGT RNDIAIIWPP KGDPVVLAVL SSRDKKDAKY
M. fo.      AGLPEGWTTA DKTGSGDYGS TNDAGIAFGP DGQRLLLVMM TRSQAHDPKA
Consensus   aGlP.dWtla DKTG.gdYGt nNDagv.wpP grppvvl.vl ttrd.pDaka

            280
S. fr.      DNV..LVAEA ARL
S. la.      DN..PLVAKT AAL
S. al.      DD..GLVADA ARV
B. li.      DDK..LIAEA TKV
M. fo.      ENLRPLIGEL TAL
Consensus   dn...Lvaea a.l
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Fig. 2. Alignment of the sequence of the *M. fortuitum* β-lactamase (M. fo) with those of other class A β-lactamases. The analysis was performed using sequences of 22 β-lactamases and methods described by Joris *et al.* (1991). Only the sequences of enzymes giving the best scores are shown. These are: *Streptomyces fradiae* (S. fr, score 49), *Streptomyces lavandulae* (S. la, score 48), *Streptomyces albus* G (S. al, score 49), and *Bacillus licheniformis* (B. li, score 45). The scores versus the various β-lactamase families were as follows: Gram-negative, 41.8±1.9; bacIII, 41.8±2.1; streptomycetes I, 43±0.8; streptomycetes II, 47±3.4. In the consensus sequence, the strictly conserved residues are in bold characters. The ABL numbering (Ambler *et al.*, 1991) is used. The active Ser residue is marked by an asterisk.

β-galactosidase activity, respectively. These results proved the base difference at position 948 (C in *blaF*, T in *blaF**) has no effect on transcription. Since *lacZ* was efficiently expressed in these constructs, we inferred that translation does indeed start at ATG position 1275.

Expression analysis

The level of β-lactamase production in *M. smegmatis* harbouring either pIPJ47(*blaF*), or pIPJ47*(*blaF**), or pRR3, was analysed by measuring the minimal inhibitory concentration (MIC) of ampicillin (Table 1), by measuring β-lactamase activity in culture supernatants (Table 1) and by immunoblot analysis using anti-BlaF antibodies (Fig. 5). These results showed that the *M. fortuitum* BlaF was synthesized and secreted in an active form in *M. smegmatis*, thereby conferring high-level ampicillin resistance. Beta-lactamase assays and immunoblot analysis confirmed that these high levels of ampicillin resistance resulted from the production of the *M. fortuitum* enzyme, although the endogenous *M. smegmatis* β-lactamase was also produced and could be visualized as a faint band in the immunoblot (lane 5 in Fig. 5, and data not shown). These analyses also demonstrated that *M. smegmatis* carrying pIPJ47* was more resistant to ampicillin and produced more β-lactamase than the strain carrying pIPJ47.

Table 1. MIC and β-lactamase activity of *M. smegmatis* clones expressing *blaF* or *blaF**.

Plasmid	MIC of ampicillin ($\mu g\,ml^{-1}$)	β-lactamase activity[a]
pRR3	10	20
pIPJ47*	100	450
pIPJ47	30	180

a. β-lactamase activity is expressed in arbitrary units calculated as described in the *Experimental procedures*.

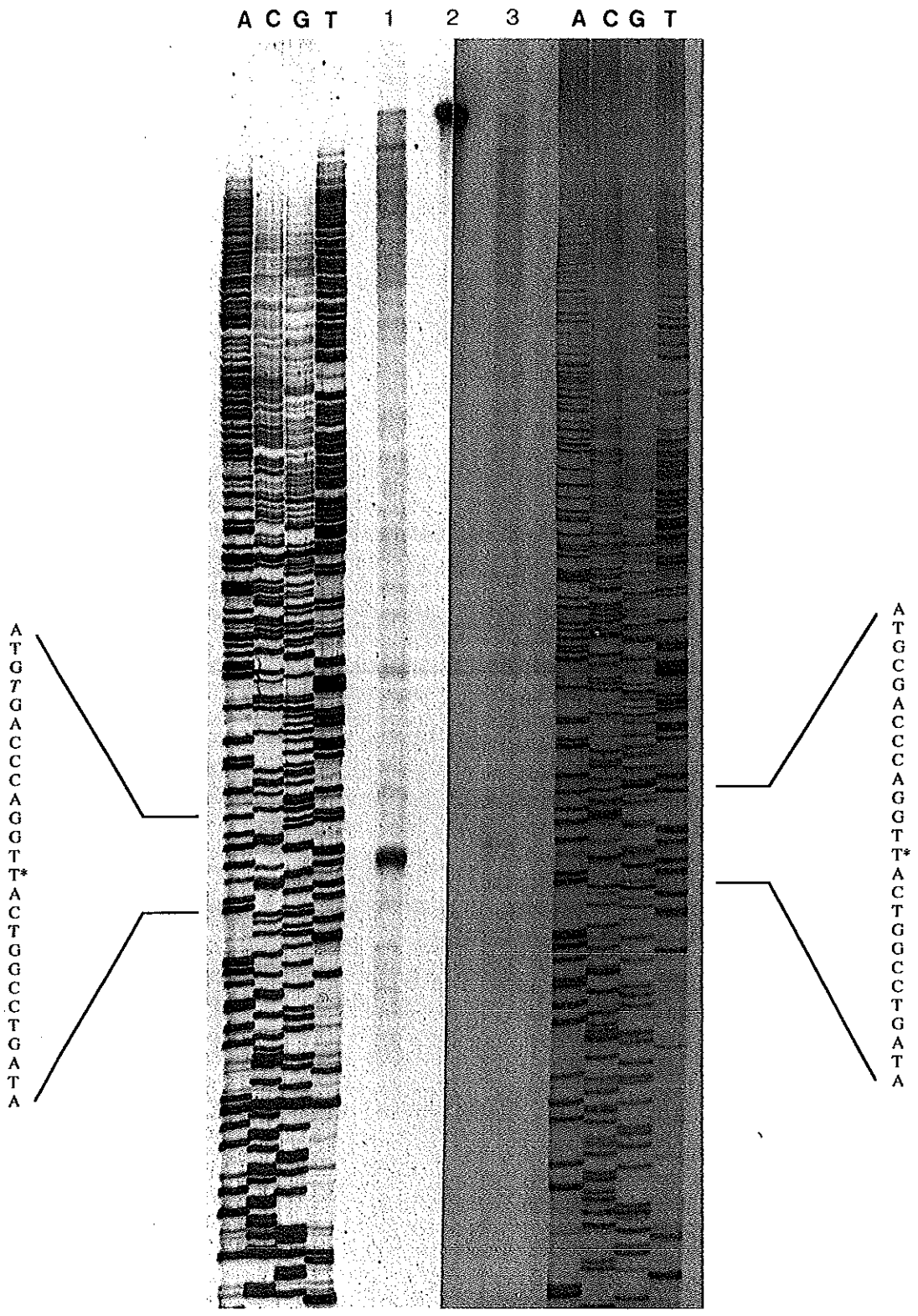

Fig. 3. S1 mapping of the *blaF* and *blaF** mRNA. Lane 1, product of the reaction performed with RNA extracted from *M. smegmatis* (pIPJ47*); 2, labelled probe; 3, product of the reaction performed with RNA extracted from *M. smegmatis*-pIPJ47. The 5′ ends of the mRNAs are indicated by asterisks. The sequences shown are the transcribed strands of *blaF** (left-hand) and *blaF* (right-hand). The base pair substitution found in *blaF** is indicated by an italicized letter. The right-hand part of the autoradiography was overexposed to enable visualization of the faint signal.

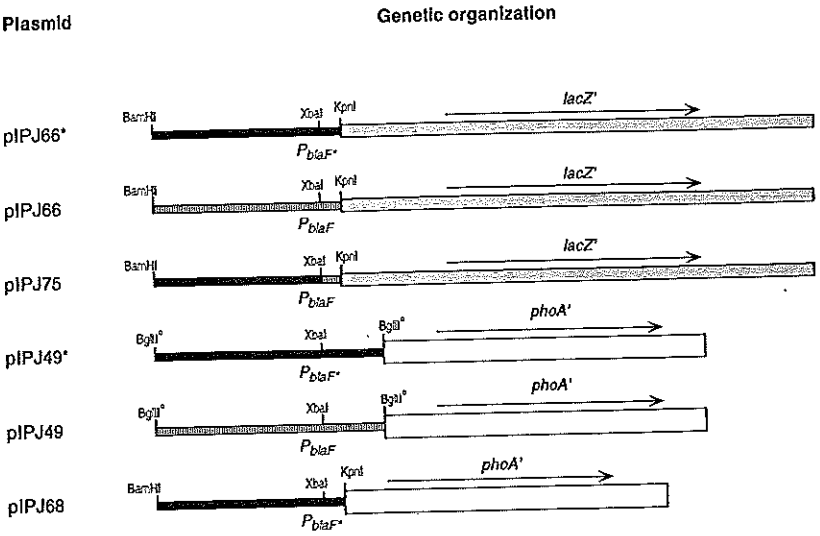

Fig. 4. Schematic representation of plasmid constructions harbouring *lacZ* or *phoA* gene fusions. Only the inserts of recombinant plasmids are shown.

Expression analysis using phoA *gene fusions*

In order to quantify the expression of *blaF* and *blaF**, a truncated *phoA* gene (Gutierrez and Devedjian, 1989) was cloned into the *Bgl*II site of plasmids pIPJ47 and pIPJ47*, resulting in plasmids pIPJ49 and pIPJ49*, respectively (Fig. 4). *phoA* was chosen as a reporter gene because the encoded protein, alkaline phosphatase, is a periplasmic enzyme from *E. coli* and thus is capable of crossing a bacterial cytoplasmic membrane (Hoffman and Wright, 1985). Moreover, its activity can be easily assayed (Brockman and Hepel, 1968), and the enzyme usually possess a high activity only when it is exported. Transformation of *M. smegmatis* with these plasmids by electroporation followed by plating on 7H10 plates containing the chromogenic phosphatase indicator 5-bromo-4-chloro-3-indoxyl phosphate (XP) resulted in PhoA$^+$ (blue) colonies within 4 d when pIPJ49* was used, and within 5 d with pIPJ49. Sonicated extracts of *M. smegmatis* harbouring pIPJ49 or pIPJ49* contained 11 and 34 units of alkaline phosphatase activity, respectively, compared with 0.3 units in extracts of control cells harbouring pRR3. Immunoblot analysis of culture supernatants and sonicated extracts of strains expressing the gene fusions using anti-PhoA antibodies (Fig. 6) unexpectedly revealed that almost all of the β-lactamase–alkaline phosphatase (BlaF–PhoA) hybrid was present in the sonicated extracts (lanes 2 and 3), with small amounts of a protein with the same M_r as PhoA present in the culture supernatants (lanes 7 and 8). From these data, it was inferred that BlaF–PhoA, which was translocated across the cytoplasmic membrane, remained attached to the surface. Cleavage of BlaF–PhoA hybrid by endogenous proteases presumably resulted in the

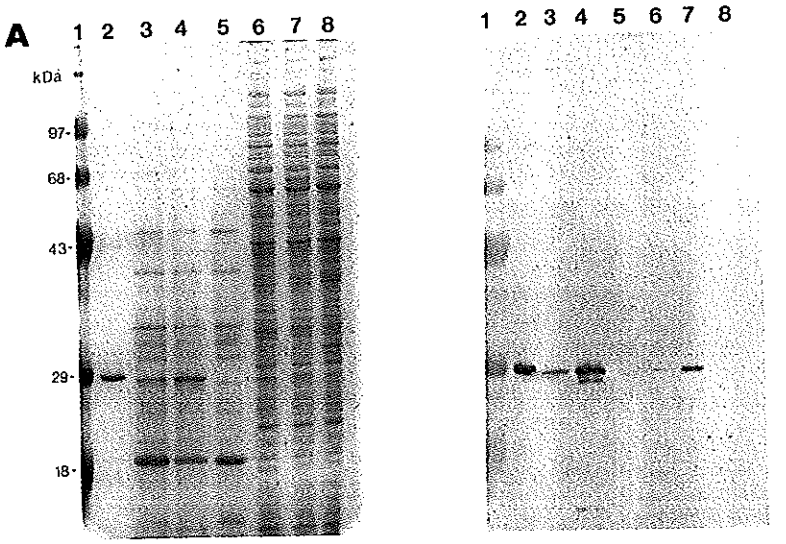

Fig. 5. Analysis of BlaF expression in *M. smegmatis*.
A. SDS–12% polyacrylamide gel stained with Coomassie brilliant blue.
B. Immunoblot using polyclonal anti-Bla antibodies. Lane 1, prestained molecular mass standards; 2, 1 μg of purified BlaF; 3, supernatant of a *M. smegmatis*(pIPJ47) culture; 4, supernatant of a *M. smegmatis*(pIPJ47*) culture; 5, supernatant of a *M. smegmatis*(pRR3) culture; 6, sonicate extract of *M. smegmatis*(pIPJ47); 7, sonicate extract of *M. smegmatis*(pIPJ47*); 8, sonicate extract of *M. smegmatis*(pRR3). Twenty-five micrograms of TCA-precipitated proteins from culture supernatants, and 50 μg of sonicated cell extracts were loaded on to the gels.

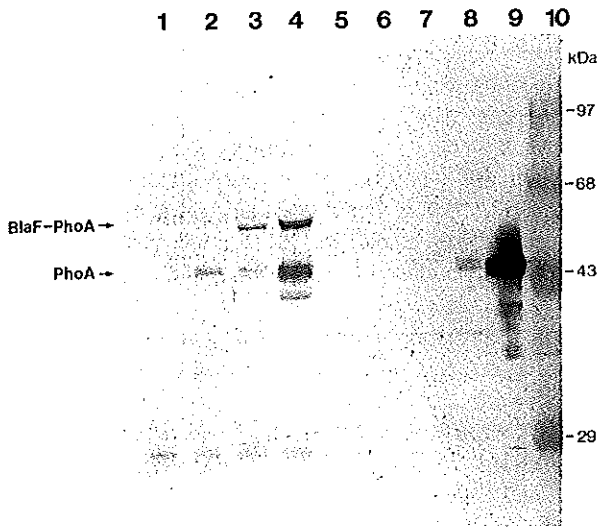

Fig. 6. Immunoblot analysis of production of BlaF–PhoA hybrids in *M. smegmatis*. Immunoblot using polyclonal anti-PhoA antibodies. Lane 1, extract of *M. smegmatis*(pRR3); 2, extract of *M. smegmatis*(pIPJ68); 3, extract of *M. smegmatis*(pIPJ49); 4, extract of *M. smegmatis*(pIPJ49*); 5, supernatant of a *M. smegmatis*(pRR3) culture; 6, supernatant of a *M. smegmatis*(pIPJ68) culture; 7, supernatant of a *M. smegmatis*(pIPJ49) culture; 8, supernatant of a *M. smegmatis*(pIPJ49*) culture; 9, 3 μg of purified PhoA; 10, prestained molecular mass standards. Fifty micrograms of TCA-precipitated proteins from culture supernatants, and 100 μg of sonicated cell extracts were loaded on to the gels.

accumulation of the PhoA segment of the hybrid detected on the immunoblot shown in Fig. 6. Proteolysis of the surface-anchored hybrid could have allowed the release of small amounts of the PhoA segment into the medium, but most of it remained cell associated.

In order to test for the requirement of the BlaF segment of the hybrid in the production of active alkaline phosphatase and for the release of the PhoA segment into the culture medium, the truncated *phoA* gene was fused directly to *blaF** start codon to give pIPJ68 (Fig. 4). *M. smegmatis* transformed with this plasmid only gave rise to white colonies on XP 7H10 plates. The PhoA polypeptide produced by these cells was detected only in the sonicated extracts and not in the medium (Fig. 6, lanes 2 and 6). These results demonstrated the need for the *N*-terminal region of BlaF for PhoA export and activity in *M. smegmatis*, and supported our hypothesis concerning the surface location of the BlaF–PhoA hybrid in cells carrying pIPJ49 or pIPJ49*.

Discussion

M. fortuitum strain D316 was derived from strain ATCC19452 by nitrosoguanidine treatment followed by selection for increased resistance to the β-lactam antibiotic amoxicillin (Fattorini *et al.*, 1989). The nucleotide sequence of the coding and flanking regions of the *blaF* gene from strain D316 (referred to herein as *blaF**) differed at 11 positions from that of the corresponding regions (*blaF*) from a natural, low-level resistant *M. fortuitum* isolate, FC1. We speculated that some of these sequence differences could explain the higher β-lactam resistance of strain D316, although others could have resulted from natural genetic drift without affecting the level of resistance (Gallieni *et al.*, 1988).

The seven sequence differences downstream from the *Bgl*II site in the coding region of *blaF*(*blaF**) (see EMBL entry L25634 for complete sequence) are mostly silent or conservative changes that probably have little if any influence on β-lactamase production or activity. Among the four sequence differences in the 5′ region upstream from the *Bgl*II site, those at positions 67 (Leu in *blaF*, Pro in *blaF**) and 81 (Ala or Val) (Fig. 1) did not affect BlaF activity (data not shown). Thus, the mismatches considered most likely to explain the higher level of resistance of D316 were those upstream of the presumed start codon: G or A at position 948 and C or T at position 1265. To test this, we expressed *blaF* and a *blaF**/*blaF* fusion that differed from *blaF* only at the four positions upstream from the *Bgl*II site (see Fig. 1 and Fig. 9 later) in *M. smegmatis*. Both genes conferred high-level ampicillin resistance, with the strain carrying the *blaF**/*blaF* fusion being more resistant and containing correspondingly more β-lactamase activity and BlaF protein. Furthermore, expression of *blaF–phoA* and *blaF–lacZ* gene fusions in *M. smegmatis* resulted in the production of higher levels of alkaline phosphatase or β-galactosidase activities, respectively, when the gene fusions included 5′ sequences derived from *blaF**. These data confirm that β-lactamase could play a significant role in resistance to β-lactams in a mycobacterial species, as reported by Fattorini *et al.* (1991), and that sequence differences within the regulatory regions of the two genes could explain the higher level of resistance conferred by *blaF**.

The higher level of BlaF production in strains carrying *blaF** is apparently due to a higher level of transcription. Northern blot analysis and S1 mapping experiments showed that the corresponding messenger RNA is approximately 10-times more abundant in *M. smegmatis* clones containing *blaF** sequences than in those containing *blaF*. Transcription of both forms of the gene starts at the same nucleotide, which is also the translational initiation codon. Translation of leader-less mRNA has already been observed for 11 other actinomycete genes, for the bacteriophage lambda *cI* gene transcribed from the *prm* promoter, for the *tetR* gene in transposon Tn*1721*, for bacteriorhodopsin-related genes in *Halobacterium halobium*, and for the *polA* gene of *Streptococcus pneumoniae* (Strohl, 1992).

The bases at position −10 (from the transcription/translation start site) in *blaF** and *blaF* are A and G,

Promoter	-35		-10	tss
P2*hsp60*	GCTTCTTGCACTCGGCATAGGCGAGTGC		TAAGAATAACGTTG	
P*AN*	GTGAATCGACAGGTACACACAGCCGCCA		TACACTTCGCTTCA	
P*BlaF*	AGCTTCAAACTATCGCCGGCTGACCCGC		TACGCTGGGTCCAA	
P*BlaF**			TACACT	
Streptomyces Eσ^{70}-like consensus	TTGAC(Pu)	(17 bp)	TAG(Pu)(Pu)T	
E. coli Eσ^{70} consensus	TTGACA	(17 bp)	TATAAT	

Fig. 7. Alignment of mycobacterial promoters. The transcriptional start sites (tss) as well as hexamers with the highest homology to the *E. coli* Eσ^{70} consensus are underlined.

respectively. Studies with *blaF–lacZ* gene fusions indicated that the enhanced transcription of *blaF** was due to this single difference, while the other difference upstream from the coding sequences of *blaF* and *blaF**, C or T respectively at position 948, did not influence P_{blaF} activity. Several 'up' mutations in promoters in enterobacteriaceae and coliphages result from substitutions in their −10 regions that increase their similarity to the *E. coli* σ^{70} −10 consensus (Youderian *et al.*, 1982). Thus, the sequence of the −10 hexamer of P_{blaF*}, which is identical to that found in P_{AN} (Murray *et al.*, 1992), may be closer to that of a consensus mycobacterial promoter than the P_{blaF} hexamer.

Only three mycobacterial promoters have hitherto been located on the basis of transcription start site mapping, namely *M. bovis* BCG *hsp60* P1 and P2 (Stover *et al.*, 1991), and P_{AN}. The first two control the *groEL2* gene encoding a 65 kDa heat-shock protein, while P_{AN} drives the expression of a gene of unknown function (ORF2) carried by IS*900* isolated from *Mycobacterium paratuberculosis*. The nucleotides to which the mycobacterial RNA polymerase binds in these promoter regions have not been identified. Nevertheless, it is reasonable to assume that the mycobacterial RNA polymerases, like those from other bacteria, interact mainly in the −10 and −35 regions when associated with their vegetative sigma factor (the equivalent of σ^{70} in *E. coli*; McClure, 1985). An alignment of the *hsp60* P2 and P_{AN} sequences with those of P_{blaF} and P_{blaF*} is shown in Fig. 7. BCG *hsp60* P1 was not included in this alignment as it was shown not to be recognized by the RNA polymerase from non heat-shocked cells of *M. smegmatis* (Levine and Hatfull, 1993). On the contrary BCG *hsp60* P2 is recognized by this enzyme, although it has several unusual features. Both P_{AN} and P_{blaF} (P_{blaF*}) are active in *M. smegmatis* grown at 37°C, and thus are likely to be transcribed by a σ^{70}-type RNA polymerase. −10 and −35 hexamers homologous to the consensus sequence of those in promoters recognized by *E. coli* σ^{70} (Harley and Reynolds, 1987) and the *Streptomyces* vegetative RNA polymerases (Strohl, 1992) were readily identified in all three promoters. These sequences are separated by the optimal 17 bp in *hsp60* P2 and in P_{AN}, and by the suboptimal 19 bp in P_{blaF} (P_{blaF*}). The −10 regions of P_{AN} and P_{blaF*} have four out of six bases identical to the *E. coli* −10 (σ^{70}) consensus, whereas the −10 region of BCG *hsp60* P2 is less similar to the consensus sequence (three out of six bases identical). However, this promoter is probably unrepresentative of vegetative promoters of mycobacteria since the region between its −10 and −35 sites includes an imperfect palindrome (TTCTTG-CACTC-9N-GAGTGCTAAGAA) which is similar to structures observed in the regulatory regions of chaperone-encoding genes from various organisms (Guglielmi *et al.*, 1993; P. Mazodier, personal communication). Recently, Das Gupta *et al.* (1993) provided substantial evidence supporting earlier suggestions that most mycobacterial promoters are not recognized in *E. coli* (Clark-Curtiss *et al.*, 1985; Hopwood *et al.*, 1988). P_{AN} and P_{blaF} (P_{blaF*}) may differ from other mycobacterial promoters in that they closely resemble the *E. coli* σ^{70} consensus.

Another aspect of mycobacterial gene expression in *E. coli* concerns translation efficiency. *lacZ* operon and gene fusions were recently used by Murray *et al.* (1992) to show that P_{AN} is active in *E. coli* even though ORF2 is not translated. *E. coli* transformed with plasmids bearing either of the *blaF* alleles did not become ampicillin resistant (data not shown). We speculate that *blaF* mRNA may be poorly translated owing to the absence of a prototype ribosome-binding site (RBS). RBS sequence recognition in mycobacteria may be less stringent than in *E. coli*, as is the case in *Streptomyces* (Strohl, 1992). Truncated forms of *phoA* and *lacZ* appear to be very efficiently expressed when fused directly to *blaF* (or *blaF**) start codons, i.e. in the absence of a Shine–Dalgarno sequence, in *M. smegmatis* and *M. bovis* BCG (Fig. 4 and unpublished data). Furthermore, the putative Shine–Dalgarno sequences of 12 recently sequenced *M. leprae* genes are located at varying positions with respect to the translation start site and exhibit minimal complementarity to the 3′-end of 16S mycobacterial rRNA (Honoré *et al.*, 1993).

When *M. smegmatis* carrying *blaF* or *blaF** was grown exponentially in shaken cultures in the presence of a detergent (to avoid clumping), BlaF was found mainly in the culture medium. This result and those reported by Fattorini *et al.* (1991) with *M. fortuitum* indicate that mycobacterial β-lactamases are extracellular. The *blaF* and *blaF** genes were therefore fused to the *E. coli phoA* gene to study the role of the signal peptide in BlaF secretion. The *blaF*(*blaF**)*–phoA* constructs conferred a $PhoA^+$ phenotype on *M. smegmatis* when *phoA* was cloned downstream of the segment coding for the BlaF signal peptide, but not when *phoA* was fused to the *blaF** start codon. Immunoblot analysis showed that PhoA was exclusively cell associated in the latter, $PhoA^-$ clones, and both cell-associated and extracellular in the former, $PhoA^+$ clones. Thus, BlaF is made with a functional signal peptide which is probably necessary for translocation across the membrane. The inefficient extracellular release of the BlaF–PhoA hybrid and of the PhoA polypeptide, which is probably derived from it by endogenous proteolysis, could be explained by their entrapment by the cell wall. The possibility that a relatively large proportion of this hybrid is not even exported should also be considered, although the higher level of alkaline phosphatase activity detected in extracts of strains habouring *blaF*–phoA* compared to those of strains carrying *blaF–phoA* show that the export pathway is not blocked by the BlaF–PhoA hybrid. Thus, *phoA* can be used as a reporter gene for studying protein export in mycobacteria, as it has in other bacteria (Slauch and Silhavy, 1991).

Experimental procedures

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are shown in Table 2. *E. coli* strains were grown in L-broth or on L-broth agar supplemented with the appropriate antibiotic and were transformed as described (Sambrook *et al.*, 1989). *M. smegmatis* was transformed by electroporation (Martín *et al.*, 1990b), and grown in the presence of 25 μg ml^{-1} kanamycin on one of the following media (see below): L, Beck (Gheorgiu, 1988), Middlebrook 7H9, or 7H10 (Difco).

To determine the minimal inhibitory concentration (MIC) of ampicillin, *M. smegmatis* was grown on duplicate 7H10 plates containing appropriate concentrations of the antibiotic. The MIC was defined as the concentration resulting in a 90% reduction in the number of colony-forming units detected after 48 h incubation at 37°C.

Oligonucleotides and PCR amplifications

The oligonucleotide primers were synthesized on a Cyclone DNA Synthesizer (Bioresearch). The degenerate oligonucleotide mixes Moligo1 (5′-GC(C,G)CC(G,C)ATCGA(C,T)GA-(C,T)CAG-3′) and Moligo2 (5′-AG(C,G)TG(C,G)AAGTTCC-(G,C)AT(A,G)CA(G,C)-3′) were designed using the preferential mycobacterial codon usage and corresponded, respectively, to the first six and the last seven amino acids of the *N*-terminal region of BlaF (Amicosante *et al.*, 1990). PCR amplifications were carried out in a DNA Thermal Cycler (Perkin Elmer), using *Taq* polymerase (Cetus) according to the manufacturers' recommendations.

Primers used for sequencing were based on previously determined sequences. The remaining oligonucleotides used in this study were BlaO1 (5′-CTTGAACGTCGAGCA-CATCGCGAACAT-3′) OBlaF9 (5′-TTTGAAGCTGTTGTGG-CAGCGCTT-3′), OBlaF11 (5′-GGGGTACCATTGGACCCA-GTG-3′), OBlaF12 (5′-GGGGTACCATTGGACCCAGCG-3′), OBlaF13 (5′-GGGGTACCTCGCCGTGCTCGG-3′), OBlaF14 (5′-CGGGATCCTGCTCGGCGGACTCC-3′) and OBlaF15 (5′-GAAGATCTCGAGGCGGACGAGGACAACAT-3′).

DNA manipulation

Standard recombinant DNA techniques were carried out as described (Sambrook *et al.*, 1989). For sequence determinations, DNA fragments were cloned into M13mp18 and M13mp19 bacteriophage DNA or into pUC derivatives (Yanisch-Perron *et al.*, 1985). The DNA sequence was determined on both strands using the dideoxy chain-termination method (Sanger *et al.*, 1977) and T7 DNA polymerase (Sequenase, USB; Pharmacia) or *Taq* DNA polymerase (Taq Track, Promega). To avoid errors arising during PCR amplification by *Taq* DNA polymerase, the *blaF* fragment synthesized by PCR was sequenced from two independent clones.

Construction of the M. fortuitum *gene libraries*

A genomic DNA library of *M. fortuitum* strain D316 was produced by cloning DNA partially digested with *Sph*I into pACYC184 linearized with the same enzyme and dephosphorylated with calf intestine alkaline phosphatase. This library contains approximately 2500 recombinant clones. The genomic DNA library of *M. fortuitum* strain FC1 was obtained as follows: the DNA was totally digested with *Bam*HI, and fragments between 4 and 10 kb were isolated from an agarose gel with Geneclean (Bio 101 Inc.), and cloned into *Bam*HI-cleaved and dephosphorylated pUC18. The FC1 library contains aproximately 1600 recombinant clones.

Cloning of β-lactamase genes from M. fortuitum

With the aim of cloning the β-lactamase gene from *M. fortuitum*, a DNA fragment, BlaM1, encoding the *N*-terminal region of the purified protein sequenced by Amicosante and co-workers (1990), was synthesized *in vitro* by PCR amplification. This reaction was performed using the degenerate oligonucleotide mixes Moligo1 and Moligo2 as primers, and total DNA from *M. fortuitum* FC1 as a template. The product of the PCR reaction was cloned into plasmid pUC18 and sequenced. From this sequence, an oligonucleotide, BlaO1, was designed and used as a probe to screen a genomic DNA library of *M. fortuitum* D316 by colony hybridization. A clone hybridizing to BlaO1 was isolated and analysed. This clone carried a

Table 2. Strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics	Source/Reference
M. fortuitum D316	High-BlaF-producing mutant	Fattorini *et al.* (1989)
M. fortuitum FC1		Zaragoza collection
M. smegmatis mc^{2}155	High-transformation mutant of *M. smegmatis* ATCC607	Snapper *et al.* (1990)
E. coli MC1061	F$^-$ *araD319* Δ(*ara leu*)*7696* Δ*lacX74 galU*$^-$ *galK*$^-$ *hsr*$^-$ *hsm*$^+$ *strA*	Sambrook *et al.* (1990)
E. coli XL1-Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]	Sambrook *et al.* (1990)
E. coli HB101	*supE44 hsdS20* (r$^-$m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	Sambrook *et al.* (1990)
pACYC184	Cloning vector	Rose *et al.* (1988)
pUC18	Cloning vector	Yanish-Perron *et al.* (1985)
pSL1180	Cloning vector	Brosius *et al.* (1989)
pPHO7	pUC derivative carrying a truncated *phoA* gene	Gutierrez *et al.* (1989)
pRR3	*E. coli*-mycobacteria shuttle vector	Ranes *et al.* (1990)
pBF1	pACYC184 derivative carrying a truncated *blaF**	This work
pIPJ39	pUC derivative carrying *blaF*	This work
pIPJ42	pUC derivative carrying *blaF*	This work
pIPJ42*	pUC derivative carrying a *blaF–blaF** fusion	This work
pIPJ46	pSL1180 derivative carrying the ORIM*aph* cassette	This work
pIPJ47	pUC derivative carrying *blaF*	This work
pIPJ47*	pUC derivative carrying a *blaF–blaF** fusion	This work
pIPJ49	pUC derivatives carrying a *blaF–phoA* fusion	This work
pIPJ49*	pUC derivative carrying a *blaF*–phoA* fusion	This work
pIPJ66	pRR3 derivative carrying a *blaF–lacZ* fusion	This work
pIPJ66*	pRR3 derivative carrying a *blaF*–lacZ* fusion	This work
pIPJ68	pRR3 derivative carrying a *blaF*–phoA* fusion	This work
pIPJ71	pUC derivative carrying the *C*-terminal fragment 365 bp of *blaF**	This work
pIPJ75	pRR3 derivative carrying a *blaF–lacZ* fusion	This work

recombinant plasmid, pBF1, containing an insert of approximately 4.7 kb. A restriction map of this plasmid was constructed and the region hybridizing with BlaO1 was shown to be located at one end of the insert (Fig. 8). Nucleotide sequencing of this region revealed the presence of an ORF encoding a polypeptide homologous to class A β-lactamases. However, this ORF appeared to be incomplete because the encoded polypeptide is smaller than known β-lactamases.

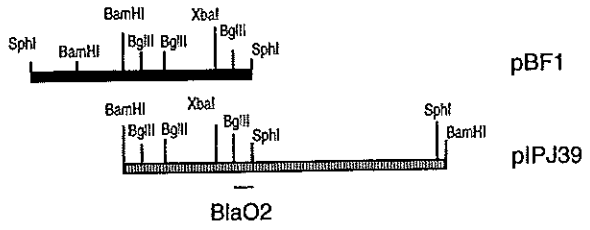

Fig. 8. Alignment of the restriction maps of the inserts in pBF1 and pIPJ39. The BlaO2 probe (underlined region) was prepared from a *BglII–SphI* fragment of 390 bp.

In order to isolate the complete gene, another genomic library, constructed with DNA from *M. fortuitum* FC1, was screened with a probe, BlaO2, corresponding to an intragenic fragment of pBF1 (Fig. 8). Three clones hybridizing with BlaO2 were obtained. One of these clones contained a plasmid, pIPJ39, with an insert of approximately 7 kb. This clone was selected for further investigation. Analysis of the restriction map of pIPJ39 suggested that it contained the entire *M. fortuitum* FC1 β-lactamase gene, *blaF*, since the region hybridizing to BlaO2 was at the centre of the insert (Fig. 8). Nucleotide sequencing of this region confirmed this prediction.

For unknown reasons attempts at cloning the missing 3′ extremity of the *M. fortuitum* D316 β-lactamase gene, *blaF**, from the corresponding genomic DNA library were unsuccessful. We therefore decided to synthesize this fragment by PCR, using the D316 genomic DNA preparation as a template and oligonucleotides hybridizing to the 3′ end of *blaF* (OblaF9 and 15) as primers. The PCR product was cloned into pUC18, resulting in pIPJ71.

Plasmid constuction

The construction of pIPJ39 and pBF1 is described above. pIPJ46 is a derivative of pSL1180, which contains the origin of replication (*Eco*RV–*Kpn*I fragment) of the *M. fortuitum* plasmid pAL5000 (Ranes *et al.*, 1990) and the kanamycin-resistance gene from Tn*903* (Kanamycin Resistance GenBlock (Pharmacia)) inserted between its *Eco*RV and *Kpn*I sites. The construction of pIPJ42, pIPJ42*, pIPJ47, and pIPJ47* is illustrated in Fig. 9. These plasmids contain different fragments inserted into the *Bam*HI site of pUC18. pIPJ49 and pIPJ49* were obtained by cloning the *Bam*HI fragment of pPHO7 (corresponding to the truncated *phoA* gene) into the *Bgl*II site of pIPJ47 and pIPJ47*, respectively. pIPJ66, pIPJ66* and pIPJ68 contain the PCR-amplified fragments A and B, corresponding to the regulatory sequences of *blaF* (or *blaF**) and their start codons (1277 bp), fused to the *Kpn*I sites of the truncated *lacZ* or *phoA* genes. Fragments A and B were synthesized using the following pair of primers: A (OBlaF11 and 14) and B (OBlaF12 and 14). They were sequenced to verify the absence of errors arising during PCR amplification.

RNA manipulation

RNA extraction and transcript mapping were performed using methods compiled by Hopwood *et al.* (1985), with previously described modifications (Murray *et al.*, 1992). The DNA fragment used as a probe in the S1 mapping experiments was obtained by digesting pIPJ42 with *Bgl*II, dephosphorylation, and redigestion with *Sph*I. The 4.4 kb DNA fragment was isolated from an agarose gel with Geneclean and labelled with [γ-^{32}P]-ATP (3000 Ci mmol^{-1}) using T4 polynucleotide kinase. Unincorporated radioactivity was removed by passage through a Nick column (Pharmacia). Forty micrograms of each RNA preparation and 0.1 μg of the radiolabelled probe were used in each experiment.

Enzyme assays

Beta-lactamase activity was assayed by the method of O'Callaghan *et al.* (1972), with some modifications, using culture supernatants of *M. smegmatis* grown in Beck medium at 37°C for 48 h. Briefly, cell cultures were centrifuged at 5000 × *g* for 10 minutes, and supernatants were diluted in 25 mM Tris HCl buffer (pH 7.5) to a final volume of 1 ml. Fifty microlitres of a nitrocefin solution (200 μg ml^{-1} in 25 mM Tris

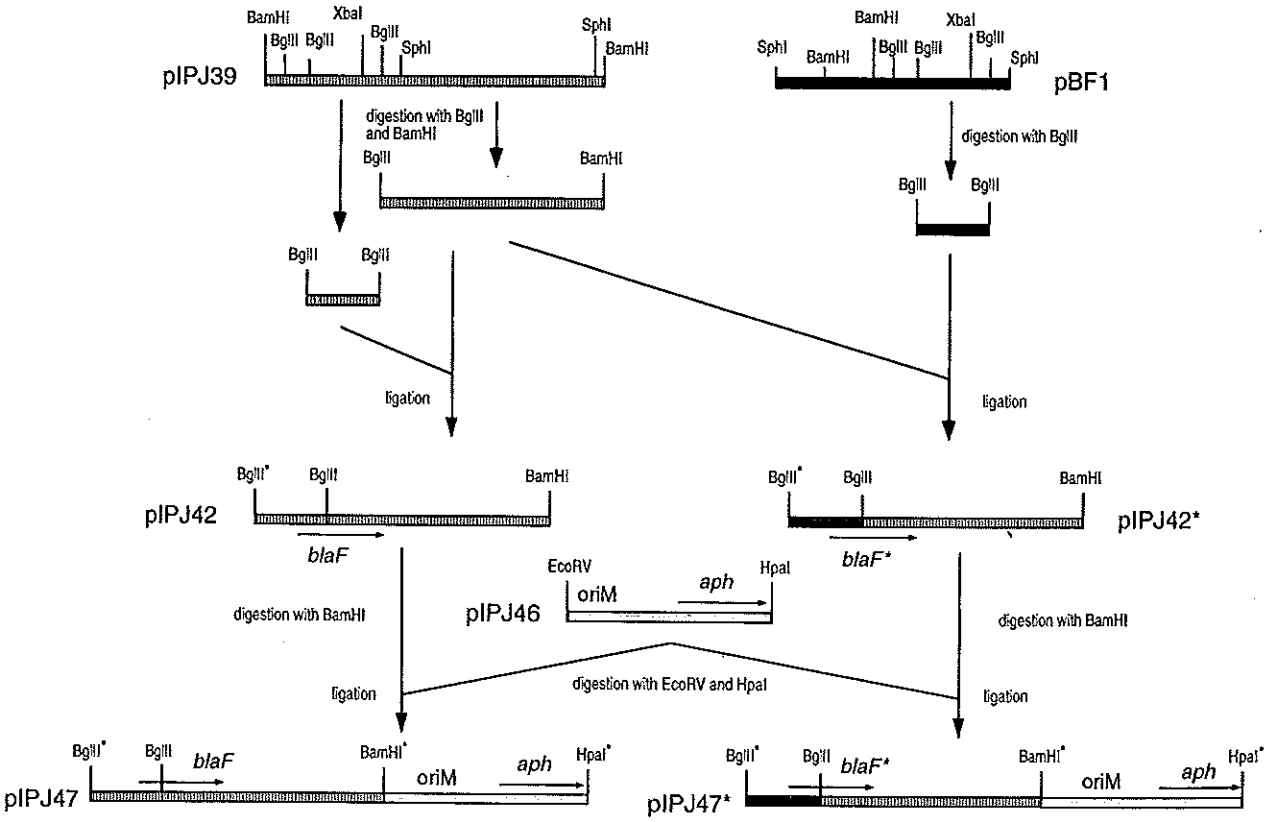

Fig. 9. Schematic representation of plasmid constructions. Only the inserts of recombinant plasmids are shown.

HCl buffer pH 7.5) were added, and the reaction allowed to proceed at room temperature until terminated by the addition of 100 µl of 1% SDS. Units of β-lactamase were calculated as $A_{490} \times 10^3$ µg protein^{-1} min^{-1}.

For alkaline phosphatase assays, *M. smegmatis* was grown in L-broth, supplemented with 0.05% tylaxopol (Sigma) at 37°C for 48 h. Alkaline phosphatase activity was assayed by the method of Brockman and Heppel (1968), with some modifications, in sonicated extracts prepared as described previously (Ranes *et al.*, 1990). Briefly, sonicated cell suspensions were diluted in distilled water to a final volume of 800 µl. One-hundred microlitres of a *p*-nitophenylphosphate solution (10 mg ml^{-1} in 1 M Tris HCl pH 9) were added, and the reaction incubated at 37°C until being terminated by the addition of 100 µl of 10 N NaOH. Units of alkaline phosphatase were calculated as $A_{420} \times 10^5$ µg protein^{-1} min^{-1}.

Beta-galactosidase assays were performed as described previously (Winter *et al.*, 1991). The amount of soluble protein contained in the extracts and supernatants was measured by using the Bio-Rad Assay (Bio-Rad). Results of the enzyme assays are the mean values resulting from two independent experiments.

SDS–polyacrylamide gel electrophoresis and immunoblots

For SDS–PAGE and immunoblot analysis, *M. smegmatis* was grown in Beck medium at 37°C for 48 h. Proteins in culture supernatants were precipitated with 10% trichloroacetic acid (TCA), and extracts prepared by sonication as described previously (Winter *et al.*, 1991). SDS–PAGE and immunoblotting were performed according to Winter *et al.* (1991).

Antibody preparations

Five-hundred micrograms of chromatographically purified PhoA (Sigma) was dissolved in 1 ml of a phosphate-buffered saline (PBS)/Freund's incomplete adjuvant (FIA) (Difco) suspension and injected subcutaneously into two New Zealand rabbits. The rabbits were boosted with the same amount of protein in PBS/FIA after 3 weeks, and bled after an additional 2 weeks. For immunoblotting, these polyclonal anti-PhoA antibodies were used at a 1:1000 dilution.

Polyclonal anti-BlaF was prepared as follows: two New Zealand rabbits were inoculated intravenously on days 0, 7, 14 and 21, with a mixture of 1 mg of purified BlaF in 1 ml PBS, emulsified with 1 ml FIA and 2 ml of PBS/2% Tween 80. Animals were bled on day 28. From this anti-serum, anti-BlaF antibodies were then semi-purified on a membrane containing purified BlaF. Briefly, 20 µg of the protein were run on a 0.1% SDS–10% polyacrylamide gel and electrotransferred to an Immobilon membrane (Milipore). This membrane was incubated with 200 µl of the anti-serum overnight at room temperature and then washed three times with PBS/0.1% Tween/0.3% skimmed milk powder and once with PBS/0.1% Tween. Seven-hundred microlitres of a 0.2 M glycine solution (pH 2.3) were added and after 5 min on ice, the eluted anti-BlaF antibodies were removed and neutralized with 40 µl of 0.3 M Tris HCl (pH 7). For immunoblotting, these anti-BlaF antibodies were used at a 1:250 dilution.

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