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Cloning and Sequence Analysis of a Class A β-Lactamase from *Mycobacterium tuberculosis* H37Ra

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A cosmid library from *Mycobacterium tuberculosis* H37Ra was introduced into *Mycobacterium smegmatis*, and eight recombinant clones with increased resistance to cefoxitin were identified. Isoelectric focusing detected an *M. tuberculosis*-derived β-lactamase in one of these recombinant clones. A sequence analysis identified it as a class A β-lactamase whose expression correlated with the increased resistance phenotype.

With the advent of multidrug-resistant *Mycobacterium tuberculosis* strains, new and effective therapies are rapidly needed to combat infections caused by these strains. There has been little incentive to examine the antimycobacterial effect of β-lactam antibiotics because (i) they were initially found to be ineffective in the treatment of tuberculosis (1) and (ii) *M. tuberculosis* was shown to produce a β-lactamase (16). However, in more recent decades, β-lactamase-stable drugs have been designed and β-lactam–β-lactamase inhibitor combinations have been shown to be effective against *M. tuberculosis* in in vitro susceptibility assays (4, 5, 26, 28). Whether the in vitro susceptibility data are predictive of clinical efficacy is not clear since clinical studies have been inconclusive (22, 29). This class of drugs needs to be reexamined for its potential role in the treatment of patients with tuberculosis.

Knowledge of the mechanisms that contribute to the resistance to β-lactam antibiotics of *M. tuberculosis* is critical for assessing their potential efficacies. The enzyme is of molecular class A (2) and has been classified as a group 2b β-lactamase according to the scheme of Bush et al. (3). As such, it has penicillinase and cephalosporinase activity which is inhibited by the β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam (14, 15, 30). We have previously shown that β-lactam antibiotics can penetrate the *M. tuberculosis* cell wall and bind their target proteins, the penicillin-binding proteins, at serum-achievable concentrations. In addition, we have demonstrated that the MICs of ampicillin, amoxicillin, cefoxitin, and ceftriaxone were all reduced when clavulanate or sulbactam was added to the regimen (6). Thus, it is quite evident that β-lactamase is the critical element of β-lactam resistance in *M. tuberculosis*. In this study we report the cloning and sequence analysis of a class A β-lactamase from *M. tuberculosis* H37Ra.

(The results of this study were presented in part at the 96th General Meeting of the American Society for Microbiology, New Orleans, La., May, 1996.)

M. tuberculosis H37Ra (ATCC 25618) was grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5% bovine serum albumin, 0.2% glucose, 0.0003% catalase, and 0.2% glycerol (6). *Escherichia coli* χ2319 was grown in 1% tryptone–0.5% yeast extract–0.5% NaCl–0.4% maltose (TYM) supplemented with 40 μg of thymidine per ml. Selection of the mycobacterium/*E. coli* shuttle vector, pYUB18 (13), in either *Mycobacterium smegmatis* mc^{2}155 or *E. coli* χ2319 was with kanamycin. (*M. smegmatis* mc^{2}155 and *E. coli* χ2319 were both kindly provided by Lolita Ramakrishna.) *E. coli* DH5α (Gibco-BRL, Gaithersburg, Md.) and pBluescript SK$^-$ (Stratagene, San Diego, Calif.) were used for subcloning by standard methods (24).

A cosmid library from *M. tuberculosis* H37Ra was constructed with pYUB18 as previously described (13). Briefly, genomic DNA was partially digested with *Sau*3AI and electrophoresed overnight in a 0.4% agarose gel. High-molecular-weight fragments (35 to 45 kb) were gel purified and ligated to *Bam*HI-digested pYUB18. The ligation mixture was packaged in vitro (Gigapack-II XL; Stratagene) according to the manufacturer's instructions except that TMGS buffer (10 mM Tris [pH 7.4]–10 mM $MgSO_4$–0.1% gelatin–100 mM NaCl) (12) was used as the diluent. After transduction of *E. coli* χ2819, purified recombinant shuttle phagemids were obtained by CsCl gradient centrifugation (24) and used for transformation of *M. smegmatis* mc^{2}155.

To obtain electroporation-competent bacteria, a 2-day-old culture of *M. smegmatis* mc^{2}155 was pelleted, washed, and resuspended in 10% glycerol. One to five micrograms of the purified cosmid DNA was mixed with 50 μl of competent cells in a cuvette (0.2-cm gap; Bio-Rad, Richmond, Calif.). Electroporation parameters were 25 kV, 25 μF, and 1,000 Ω, with a time constant of ~20 ms. The transformants were selected on plates containing 10 μg of kanamycin per ml.

Approximately 3,000 transformants were obtained after electroporation of the recombinant phagemids into *M. smegmatis*. These 3,000 transformants and the parent strain containing only the cloning vector were replica plated onto TYM plates containing 10 μg of kanamycin per ml plus 32, 64, 128, or 256 μg of cefoxitin per ml. The MIC of cefoxitin for *M. tuberculosis* H37Ra is >16 μg/ml, a value that is reduced to 8 μg/ml when the β-lactamase inhibitor clavulanic acid is present. Because clavulanic acid has no activity of its own against *M. tuberculosis* (MIC >16 μg/ml) (6), the resistance is most likely due to the hydrolysis of cefoxitin by the enzyme. Therefore, we hypothesized that transformants that expressed β-lactamase from both *M. smegmatis* and *M. tuberculosis* would show an increased resistance to cefoxitin compared to the *M. smegmatis* parent strain.

The parent strain and the vast majority of the recombinants were inhibited by 64 μg of cefoxitin per ml. Eight colonies grew at each of the higher concentrations and were considered candidates for expression of the cloned *M. tuberculosis* β-lactamase. One of the recombinant strains, IU17, was selected for further study.

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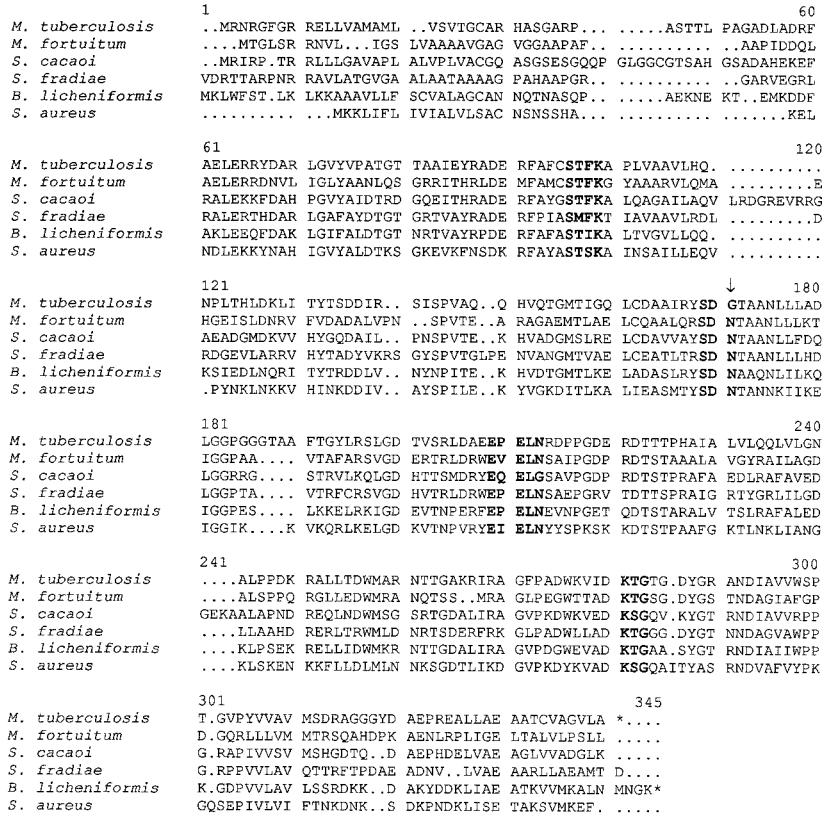

FIG. 1. Alignment of class A β-lactamases. *M. tuberculosis* β-lactamase (GenBank accession number, U67924) was aligned with β-lactamase sequences from *M. fortuitum* (GenBank accession number, L25634), *S. cacaoi* (GenBank accession number, X15708), *S. fradiae* (GenBank accession number, D13898), *B. licheniformis* (GenBank accession number, V00093), and *S. aureus* (GenBank accession number, X04121) by using the PILE-UP software program from the University of Wisconsin Genetics Computer Group package. The penicillin binding motifs found in class A β-lactamases are in boldface.

All mycobacterial β-lactamases investigated thus far have isoelectric points (pIs) between 4.4 and 6.0 (30). By adjusting the ampholyte concentrations in an isoelectric focusing (IEF) gel, the IEF pattern for *M. smegmatis* β-lactamase, having pIs of 4.3 and 5.4 (18, 21, 30), can be readily distinguished from that for the *M. tuberculosis* enzyme, having pIs of 4.9 and 5.1 (30, 31). β-Lactamase IEF patterns from recombinant strain IU17, the *M. smegmatis* host, and *M. tuberculosis* were compared. Cell extracts were obtained as described earlier (6), and equivalent protein amounts were electrofocused for 900 V · h in a 4% acrylamide–0.125% bisacrylamide matrix containing 5% ampholytes (Biolyte 3-5; BioRad) (23). Gels were incubated with 0.5 μM nitrocefin, a chromogenic substrate of β-lactamase (Cefinase; BBL). The nitrocefin stain readily diffuses in this low-acrylamide matrix; therefore, the gels were observed immediately for the detection of IEF β-lactamase patterns from *M. smegmatis* or *M. tuberculosis.*

Recombinant strain IU17 had an IEF pattern that was a composite of the patterns found in *M. tuberculosis* and *M. smegmatis*, suggesting that it expressed a β-lactamase from both. β-Lactamase activity was not detected when the cosmid was in the *E. coli* host.

To localize the *M. tuberculosis* β-lactamase gene on the 36-kb insert of pIU17, *Bam*HI-digested fragments of pIU17 were ligated to pYUB18 and reintroduced into *M. smegmatis*. One subclone, IU1701, exhibited increased resistance to cefoxitin and had a β-lactamase IEF pattern similar to that of IU17; i.e., nitrocefin-stained bands from both *M. tuberculosis* and *M. smegmatis* were present. The insert from strain IU1701 was subcloned into pBluescript for sequencing by the dideoxy chain termination reaction (25) with Sequenase 2.0 and universal primers. The sequencing of the entire gene on both strands was subsequently performed at the Biomedical Resource Center at the University of California, San Francisco.

The sequence obtained was analyzed with the University of Wisconsin Genetics Computer Group software package (8). The amino acid sequence translated from one of the open reading frames contained the penicillin binding motifs that are found in all proteins of the penicillin binding superfamily (9). By using the software program PILEUP, these motifs, SXXK, SDN, EXELN, and KT(S)G, were aligned with those from other class A β-lactamases (Fig. 1) (3).

Interestingly, the SDN penicillin binding motif was not completely conserved in *M. tuberculosis* H37Ra's β-lactamase. Instead, this strain's motif was SDG. This was confirmed by sequencing both strands of the subcloned gene (*blaA*) as well as the gene on the original cosmid, IU17. To rule out a cloning artifact during the original library construction, the gene was PCR amplified from strain H37Ra's genomic DNA and subjected to cycle sequencing (AmpliCycle sequencing kit; Perkin-Elmer Corp., Foster City, Calif.). Again, the motif was SDG, not SDN. This change in this motif has not been identified in any other class A β-lactamase whose sequence has been submitted to GenBank or previously reported (3, 9).

(The *blaA* sequence from *M. tuberculosis* H37Rv [gene MTCY49.07c] has recently become available as part of the *M. tuberculosis* sequencing project [GenBank accession number, Z73966]. This gene's nucleotide sequence is identical to that of

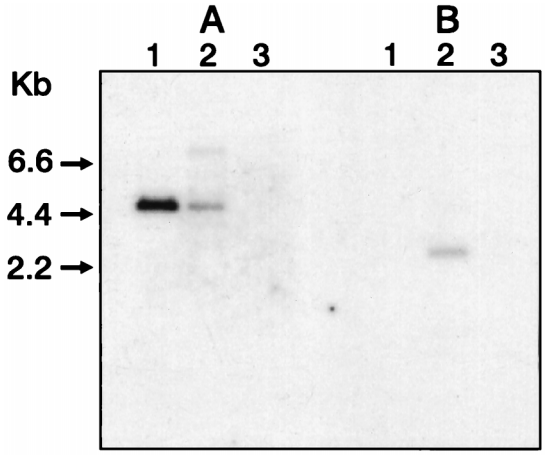

FIG. 2. Southern blot hybridization. DNA was digested with *Bam*HI, electrophoresed, and transferred to a nylon membrane. (A) Hybridization with *blaA* probe; (B) hybridization with *blaC* probe. Lanes 1, recombinant cosmid pIU17 (0.1 μg); lanes 2, *M. tuberculosis* H37Ra (5 μg); lanes 3, *M. smegmatis* mc^2155 (5 μg). Fragment sizes (in kb) are indicated on the left. The autoradiograph was reproduced digitally with Adobe Photoshop imaging software (Adobe Systems Inc., Mountain View, Calif.).

the *blaA* gene from strain H37Ra [including the unusual SDG motif described above].)

Because *M. smegmatis* contains a β-lactamase whose gene has not yet been cloned (17), the origin of *blaA* was verified by Southern blot hybridization (24). Genomic DNA from *M. tuberculosis* and *M. smegmatis* (5 μg) and from pIU17 (0.1 μg) was restriction digested, separated by agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N; Amersham). An oligonucleotide specific for the *blaA* gene (5′-GACCGGGACCGGTGACTACGG) was synthesized and 5′ end labeled with [γ-^{32}P]ATP. Hybridization and washes were at high stringency, and detection was by autoradiography. Restriction-digested fragments of the same size from pIU17 and *M. tuberculosis* genomic DNA contained the *blaA* gene (Fig. 2A). The specific probe did not hybridize with genomic DNA from *M. smegmatis*, even at low stringency; thus, *blaA* originated from *M. tuberculosis.*

The *M. tuberculosis* genome project also identified a partial open reading frame (GenBank accession number, Z73101; gene MTCY31.35) that has similarities with class C β-lactamases, such as the AmpC cephalosporinases found in gram-negative organisms (3).

To assess whether the gene for this putative β-lactamase (*blaC*) is also found in strain H37Ra, an oligonucleotide derived from the *blaC* sequence (5′-CATGCGGTAACCGACA ACGT) was constructed and used as a hybridization probe for Southern blots. The *blaC* probe hybridized with genomic DNA from strain H37Ra but not with that from pIU17 (Fig. 2B), indicating that the gene is also present in this strain but is not in close proximity to *blaA*. Just as with the *blaA* probe, hybridization with genomic DNA from *M. smegmatis* was not detected, even at low stringency. If *M. smegmatis* also has a *blaC* gene, its sequence is not very similar to that of *blaC* from *M. tuberculosis.*

It is not clear if the class C β-lactamase is produced by *M. tuberculosis*. Class C β-lactamases are typically poorly inhibited by clavulanic acid, yet we and others have found that β-lactamase activity in *M. tuberculosis* crude cell extracts is readily inactivated by this inhibitor (6, 30). Also, the reported pIs for group 1 cephalosporinases (class C) are primarily basic (3), and we have never detected β-lactamase activity in that pH range in our IEF gels.

Strain H37Ra and its parent, H37Rv, differ in their susceptibilities to ceftriaxone (MICs, 1 and 16 μg/ml, respectively) (6). Since their class A β-lactamase sequences are identical at the nucleotide level, alterations in the structure of that enzyme are probably not the cause of the difference. Whether the difference in susceptibility is due to alterations in or overexpression of the class C β-lactamase has not been determined.

Nothing is known about the regulation of *M. tuberculosis* β-lactamase production, although it has long been believed to be constitutively produced (17). Zhang et al. (30) reported that *M. tuberculosis* β-lactamase production is increased after exposure to carbenicillin, but the mechanism responsible for this is unknown.

At the amino acid level, the *M. tuberculosis* class A β-lactamase has 61% homology with that of *Mycobacterium fortuitum*, 60% homology with that of *Streptomyces fradiae*, and 62% homology with that of *Streptomyces cacaoi* (Fig. 1) (7). It has been shown that the *S. cacaoi* β-lactamase is inducible and that two regulatory genes are located upstream from the β-lactamase gene (20, 27). These two genes encode proteins similar to those identified in cephalosporinase regulatory systems in enterobacteria (19). A homologous sequence upstream of the *M. tuberculosis* β-lactamase genes would certainly suggest this type of regulation; however, none was found upstream of either the class A or class C β-lactamase genes.

The sequence homology between the class A *M. tuberculosis* β-lactamase and the class A β-lactamases found in *Bacillus licheniformis* (60% homology) and *Staphylococcus aureus* (52% homology) was also striking (Fig. 1). In these organisms there are two genes (*blaI* and *blaR1*) located upstream of the β-lactamase gene, and products from both genes are required for the regulated expression of β-lactamase (10, 11). The presence of *blaI* and *blaR1* homologs upstream of the *M. tuberculosis* β-lactamase gene would suggest this type of regulatory mechanism, but again, none was found. It is also possible that β-lactamase production in *M. tuberculosis* is regulated by some as yet unspecified system that does not involve genes directly upstream from the β-lactamase gene. However, we have been unable to detect β-lactamase induction at all in vitro. This could be because the enzyme is constitutively produced or because the β-lactam inducer was not stable over the long incubation time required for growth of *M. tuberculosis.*

Nucleotide sequence accession number. The nucleotide sequence corresponding to the class A β-lactamase sequence reported in this paper has been submitted to the GenBank database and has accession number U67924.

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