

Characterization of the Chromosomal Aminoglycoside 2'-N-Acetyltransferase Gene from *Mycobacterium fortuitum*

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A novel gene encoding an aminoglycoside 2'-N-acetyltransferase (AAC) was cloned from *Mycobacterium fortuitum*. DNA sequencing results identified an open reading frame that we have called *aac(2')-Ib* encoding a putative protein with a predicted molecular mass of 24,800 Da. The deduced AAC(2')-Ib protein showed homology to the AAC(2')-Ia from *Providencia stuartii*. This is the second member of a subfamily of AAC(2')-I enzymes to be identified. No homology was found with other acetyltransferases, including all of the AAC(3) and AAC(6') proteins. The *aac(2')-Ib* gene cloned in a mycobacterial plasmid and introduced in *Mycobacterium smegmatis* conferred resistance to gentamicin, tobramycin, dibekacin, netilmicin, and 6'-N-ethylnetilmicin. DNA hybridization with an intragenic probe of *aac(2')-Ib* showed that this gene was present in all 34 strains of *M. fortuitum* tested. The universal presence of the *aac(2')-Ib* gene in *M. fortuitum* was not correlated with any aminoglycoside resistance phenotype, suggesting that this gene may play a role in the secondary metabolism of the bacterium.

The genus *Mycobacterium* includes major human pathogens, such as the slowly growing species *Mycobacterium tuberculosis*, as well as rapidly growing species ubiquitous in the environment, such as *Mycobacterium fortuitum*, which may be opportunistic in compromised hosts. The mechanisms of resistance to antimicrobial agents found in mycobacteria are not very different from those found in other bacteria (9, 20). In fast-growing mycobacteria, several antibiotic resistance genes have been described. The gene *sul3* was detected in one strain of *M. fortuitum*. This gene encodes resistance to sulfonamide and is highly related to other *sul* genes from gram-negative bacteria (19). Tetracycline resistance determinants similar to those found in *Streptomyces* spp. and gram-positive bacteria have also been described (21). In *M. fortuitum*, β -lactamases have been described as universally present, and the gene encoding a class A β -lactamase conferring resistance to β -lactam antibiotics has been cloned (31).

Bacterial resistance to aminoglycosides is frequently mediated through aminoglycoside-modifying enzymes. These enzymes are frequently plasmid encoded, but in some cases, chromosomally encoded genes have been described (29). The chromosomally encoded acetyltransferase *aac(6')-Ic* (28) and *aac(2')-Ia* (24) genes are widely spread in *Serratia marcescens* and *Providencia stuartii* species, respectively. In the antibiotic producers *Streptomyces* species, which along with mycobacteria belong to the order *Actinomycetales*, several chromosomally located aminoglycoside acetyltransferase (AAC) genes have been characterized (11, 13, 16). The function of the chromosomal AAC enzymes remains unknown. Since they are not related to the biosynthesis of and resistance to aminoglycosides, it has been suggested that they may play a role in other metabolic processes.

The *aac(2')-Ia* gene is the most studied chromosomal *aac* gene. This gene is universally present in *P. stuartii* and is normally expressed at low levels. Since it is not inducible by aminoglycosides, most strains are aminoglycoside susceptible. The culturing of *P. stuartii* in the presence of aminoglycosides allows for the isolation of mutants with an increased level of *aac(2')-Ia* expression, which confers resistance to clinically important aminoglycosides such as gentamicin and tobramycin (24). The expression of the *aac(2')-Ia* gene is regulated at the transcriptional level by several *trans*-acting regulatory factors (18, 23, 24). Recently, Payie et al. (22) have demonstrated that the AAC(2')-Ia enzyme contributes to the O acetylation of peptidoglycan in *P. stuartii*, being essential for the maintenance of peptidoglycan structure (22).

Aminoglycoside-modifying enzymes have also been described in mycobacteria. AACs are universally present in fast-growing mycobacteria without any correlation between aminoglycoside resistance and AAC activity. In these strains, a relationship between the presence of plasmids and the production of AAC enzymes was not found (12, 32), indicating that these enzymes could be chromosomally encoded. Single-step mutational frequencies are relatively high (10^{-4} to 10^{-5}) for resistance to a single aminoglycoside, and mutants showed cross-resistance to 2-deoxy-streptomycin aminoglycosides (35). In *M. fortuitum*, AAC(3) activity has been widely reported (34), but the genes which code for these enzymes were not studied further at the molecular level.

The aim of the work described here was to characterize the genetic determinants of AAC activity in *M. fortuitum* as well as determine the relatedness between the presence of these genes and the level of resistance to aminoglycosides. As a result, we present the characterization of a chromosomal AAC gene *aac(2')-Ib*, which encodes a protein similar to the AAC(2')-Ia enzyme from *P. stuartii*.

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TABLE 1. Reference strains and plasmids used in the study

Strain or plasmid	Relevant characteristics ^a	Source or reference
Strains		
<i>M. fortuitum</i> FC1K	Gm, Km, Sm; environmental isolate	Zaragoza collection
<i>M. smegmatis</i> mc ² 155	Efficient plasmid transformation mutant	30
<i>E. coli</i> XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac</i> ⁻ F'[<i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> ΔM15 Tn10(Tet ^r)]	26
<i>S. lividans</i> 1326	Efficient transformation strain	10
Plasmids		
pSUM36	Km <i>Mycobacterium</i> sp.- <i>E. coli</i> shuttle vector	1
pAC19, pAC20, pAC21, pAC23	pSUM36 with different fragments cloned from FC1K, containing <i>aac(2')-Ib</i>	This work
pAC100	pSUM36 with 1.7-kb fragment containing <i>aac(2')-Ib</i> from pAC20	This work
pAC63	pAC20 deleted <i>EcoRV</i> -HindIII	This work
pAC63*	pAC63 BamHI digested, blunted, and religated	This work
pSK6	Probe of <i>aac(3)-Ia</i> gene	19
pWP116a	Probe of <i>aac(3)-IIIa</i> gene	3
pWP7b	Probe of <i>aac(3)-IV</i> gene	5
pJM4.22	Probe of <i>aac(3)-VII</i> gene	16

^a Abbreviations: Ap, ampicillin resistance; Km, kanamycin resistance; Gm, gentamicin resistance; Sm, streptomycin resistance.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The reference strains and plasmids used in the study are listed in Table 1. Thirty-four clinical and environmental *M. fortuitum* complex isolates (including strain FC1K) were obtained from the University of Zaragoza culture collection.

Middlebrook 7H9 broth and Middlebrook 7H10 agar (Difco Laboratories, Detroit, Mich.) were used to culture the mycobacterial strains. *Escherichia coli* XL1 was cultured in brain heart infusion (Difco). All the cultures were incubated at 37°C. Kanamycin A (20 µg/ml; Sigma Chemical Co., St. Louis, Mo.) was added when necessary.

Antibiotic susceptibility testing. The MICs of the aminoglycosides were determined on Mueller-Hinton agar (Difco) by twofold dilution of antibiotics in a range of concentrations from 0.25 to 64 µg/ml. The bacterial inoculum was adjusted to 10⁻⁵ CFU/ml, and the plates were incubated at 37°C for up to 5 days.

Assay for aminoglycoside-modifying enzymes. Cell-free mycobacterial extracts were obtained by ultrasonic disruption. The aminoglycoside-modifying enzymes were detected by the phosphocellulose paper-binding technique described previously (4). Quantitation of proteins in crude extracts was carried out as described previously (17).

Genetic techniques. Both *E. coli* XL1 and *Mycobacterium smegmatis* mc²155 were transformed by electroporation. Briefly, competent cells were prepared by culturing the strains to an optical density of 0.75 and washing three times with 10% glycerol. Aliquots were snap frozen in a dry ice-ethanol bath and were stored at -80°C. Electroporation was performed with the Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) at 2.5 kV. Plates containing 20 µg of kanamycin per ml or 4 µg of gentamicin per ml were used to select the transformants.

DNA techniques. (i) Preparation and analysis of DNA. Plasmid DNA from *E. coli* XL1 was extracted as described previously (26). Mycobacterial DNA was extracted as follows: a saturated culture of *M. fortuitum* was incubated for 14 h with 1 mg of cycloserine (Sigma) per ml. The cells were pelleted and incubated for 1 h in 10 mM Tris-1 mM EDTA-25% sucrose-4 mg of lysozyme (Sigma) per ml at 37°C. Cells were lysed after the addition of 300 µl of 1% sodium dodecyl sulfate (SDS) and 400 µg of proteinase K (Sigma) per ml and incubation at 55°C for 1 h. This was followed by the addition of 120 µl of 5 M NaCl. Following three phenol-chloroform extractions, DNA was precipitated with 98% ethanol.

(ii) Molecular biology procedures. Electrophoresis, digestions, ligations, and dephosphorylations were performed as described elsewhere (26) and according to the supplier's recommendations (Boehringer Mannheim GmbH, Mannheim, Germany).

Hybridizations. Gentamicin resistance gene probes were obtained as follows: *aac(3)-Ia* as a 3-kb BamHI fragment from plasmid pSK6 (19), *aac(3)-IIIa* as a 0.5-kb ClaI-SalI fragment from plasmid pWP116a (3), *aac(3)-IVa* as a 0.8-kb SacI-SacI fragment from plasmid pWP7b (5), *aac(3)-VIIa* as a 0.7-kb NcoI-SalI fragment from plasmid pJM4.22 (16), and *aac(2')-Ib* as a 0.42-kb BamHI-PstI fragment from plasmid pAC20 (this work).

For dot blot and Southern blot hybridizations, DNA from the *Mycobacterium* strains was transferred onto nylon filters (Hybond; Amersham International plc, Buckinghamshire, England) as described previously (26). The probes were labelled by random primer labelling (Rediprime; Amersham) with [³²P]dCTP (Redivue; Amersham). Prehybridizations and hybridizations were carried out in Rapid Hybridization Buffer (Amersham) at 65°C for 30 min and 4 h, respectively. Filters were washed at 65°C twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% SDS, once with 1× SSC-0.1% SDS, and once with

0.7× SSC-0.1% SDS. When low-stringency conditions were desired, the filters were washed at 42°C. Autoradiography was carried out by exposing X-ray film (X-Omat; Eastman Kodak Co., Rochester, N.Y.) to the filters for 18 h at -80°C.

DNA sequencing. Double-stranded DNA sequencing was performed by the dideoxynucleotide chain termination method with the fmol DNA Sequencing System (Promega Corporation, Madison, Wis.) with M13 universal primers (Promega) according to the manufacturer's instructions.

Computer analysis of sequence data. Nucleotide and amino acid sequences were analyzed and compared by using the Genetics Computer Group software (7) at Centro Nacional de Biotecnología, Madrid, Spain. Databases were searched with the program Blastncbi at the National Center for Biological Information.

Nucleotide sequence accession number. The nucleotide sequence of the *aac(2')-Ib* gene has been deposited in the GenBank data library under the accession number U41471.

RESULTS

Presence of AAC enzymes in *M. fortuitum* and resistance to gentamicin. Thirty-four environmental and clinical isolates belonging to the *M. fortuitum* complex were chosen for the present study. The MICs of gentamicin varied, ranging from 2 to 16 µg/ml. Crude extracts of all 34 strains were shown to have AAC activity. Acetylation of gentamicin, tobramycin, and kanamycins A and B was found for all the strains, showing a substrate profile consistent with the presence of an AAC(3) activity.

Environmental isolate *M. fortuitum* FC1K was chosen for further studies because of its high level of AAC activity and the level of resistance to gentamicin (MIC, 16 µg/ml).

Isolation and characterization of the gentamicin resistance determinant from *M. fortuitum* FC1K. In order to clone the genetic determinant of gentamicin resistance in *M. fortuitum*, we took two different approaches: hybridization with *aac(3)* probes from different organisms and construction of *M. fortuitum* genomic libraries in different hosts (*E. coli*, *Streptomyces lividans*, and *M. smegmatis*).

No hybridization was detected between total DNA of *M. fortuitum* FC1K and probes specific for the *aac(3)-Ia*, *aac(3)-IIIa*, *aac(3)-IVa*, and *aac(3)-VIIa* genes under low-stringency hybridization conditions.

We had previously failed to obtain gentamicin-resistant clones from an *M. fortuitum* total DNA library in *E. coli* or *S. lividans*. Recently, we described the construction of a genomic library of DNA from *M. fortuitum* FC1K partially digested with *Sau3AI* in the mycobacterial shuttle vector pSUM36. This library was transformed to *M. smegmatis* mc²155, enabling us to

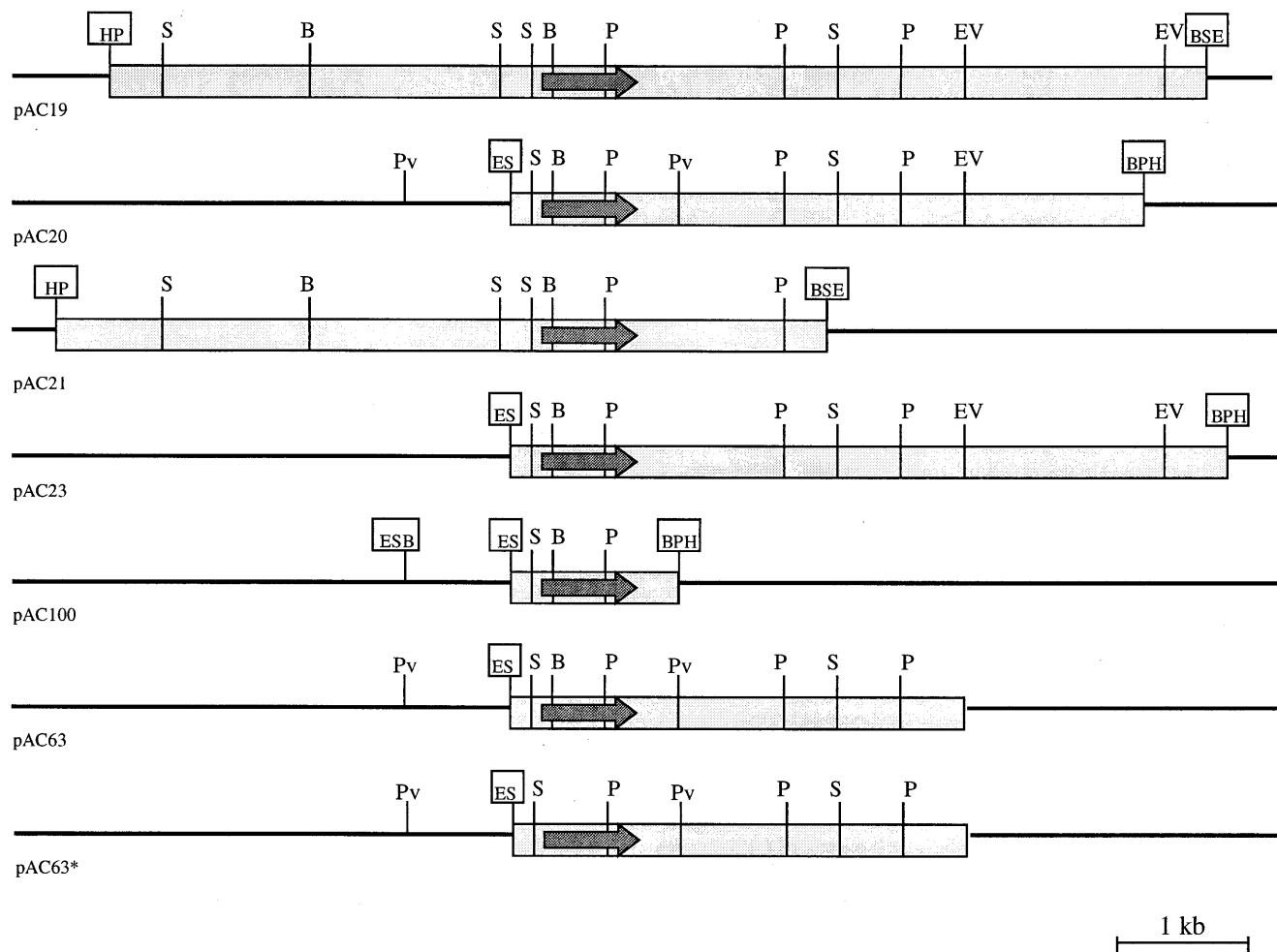


FIG. 1. Restriction maps of pAC19, pAC20, pAC21, and pAC23, four clones conferring gentamicin resistance to *M. smegmatis* mc²155 isolated from the *M. fortuitum* genomic library. The restriction sites *Sma*I (S), *Bam*HI (B), *Pst*I (P), *Eco*RI (E), *Hind*III (H), and *Eco*RV (EV) sites which mark the extremities of pAC100 are indicated; other *Pvu*II (Pv) sites present in the inserts or the vector are not shown. Sites belonging to the polylinker of pSUM36 are boxed. Grey boxes represent *M. fortuitum* DNA, and thin lines represent pSUM36 DNA. The arrow represents the extent of the *aac(2')-Ib* gene. To determine the sequence of the insert on both strands of DNA, three fragments from pAC100 were subcloned. Plasmids pAC63 and pAC63* (derivatives of pAC20) are also shown.

select 80 clones of *M. smegmatis* resistant to 4 μ g of gentamicin per ml (1). Four of these clones were analyzed and were shown to carry different recombinant plasmids, namely, pAC19, pAC20, pAC21, and pAC23, that conferred gentamicin resistance to *M. smegmatis*. Restriction maps showed that the four plasmids shared a common region (Fig. 1). Plasmid pAC20 was chosen for further studies because it carried the smallest insert. The 1.7-kb *Pvu*II-*Pvu*II fragment from pAC20 was cloned into the Klenow fragment-treated *Bam*HI site of pSUM36, resulting in pAC100. *M. smegmatis* mc²155 harboring either pAC20 or pAC100 gave an AAC(2') resistance profile, and the MICs of gentamicin, tobramycin, dibekacin, netilmicin, and 6'-*N*-ethylnetilmicin for these strains were greater than those for *M. smegmatis* mc²155 (Table 2).

DNA sequence analysis of the *aac(2')-Ib* coding region. The sequences of both strands of the insert present in plasmid pAC100 were determined. A search for stop codons in the three reading frames of each DNA strand identified an open reading frame spanning 588 nucleotides located between the GTG and TAA codons at positions 265 and 850, respectively. The likely translation start codon GTG was preceded by two possible ribosome-binding site-like sequences at positions 254

(AGAA) and 259 (AAGG). The G+C content of the *aac(2')-Ib* gene (69%) is in concordance with the values described for mycobacterial genomes (62 to 70%) (36), suggesting that this gene is indigenous to this genus.

TABLE 2. AAC(2')-Ib expression in *M. smegmatis*^a

Aminoglycosides	MIC (μ g/ml) for <i>M. smegmatis</i> mc ² 155 harboring plasmid ^b :			
	pSUM36	pAC20 or pAC100	pAC63	pAC63*
Gentamicin	0.5	16	8	0.5
Tobramycin	0.5	8	8	1
Dibekacin	1	64	32	2
Netilmicin	1	16	16	4
2'- <i>N</i> -Ethylnetilmicin	4	4	4	4
6'- <i>N</i> -Ethylnetilmicin	4	32	32	4

^a MICs of various aminoglycosides in *M. smegmatis* mc²155 harboring plasmid pSUM36 (negative control), pAC100 [pSUM36 with the *aac(2')-Ib* gene], pAC63 (derivative of pAC20), and pAC63* [*aac(2')-Ib* mutated].

^b The antibiotic concentrations ranged from 0.25 to 64 μ g/ml.

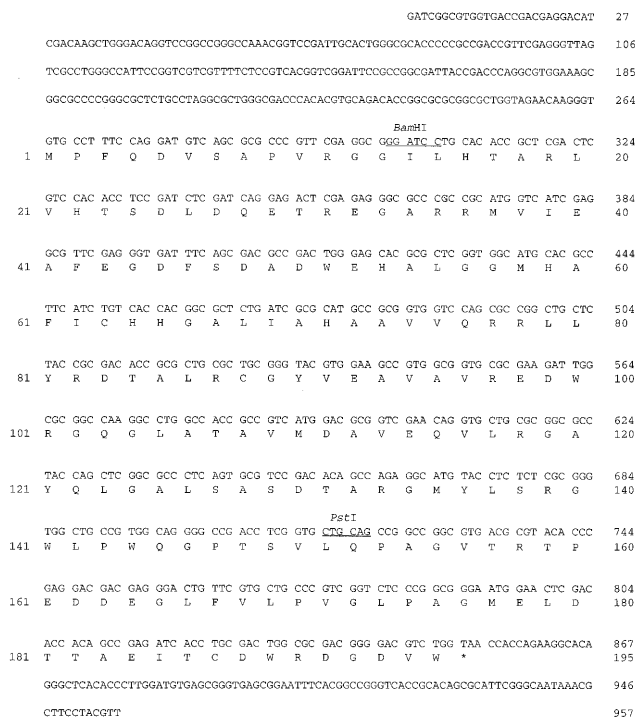


FIG. 2. Nucleotide sequence of the *aac(2')-Ib* gene and the flanking regions. Sites *Bam*HI and *Pst*I delimiting the intragenic probe used for hybridizations are underlined. The asterisk indicates the end of the protein.

The translated sequence of this open reading frame was compared with the protein sequences in protein databases, and it showed homology to the product of the gene *aac(2')-Ia* from *P. stuartii* which encodes AAC(2')-Ia. Because both enzymes share the same substrate profile, we propose the name *aac(2')-Ib* for the gene isolated from *M. fortuitum*. The region of 957 bp containing the gene *aac(2')-Ib* and the deduced sequence of the enzyme AAC(2')-Ib (195 amino acids) are presented in Fig. 2.

Inactivation of cloned *aac(2')-Ib* gene by mutation. A 1.4-kb *Hind*III-*Eco*RV fragment was deleted from plasmid pAC20, resulting in plasmid pAC63, in which the unique *Bam*HI site is located inside the gene *aac(2')-Ib*. Plasmid pAC63 was digested with *Bam*HI, blunt ended with the Klenow fragment, and religated to produce plasmid pAC63* (Fig. 1). Both plasmids pAC63 and pAC63* were electroporated in *M. smegmatis* mc²155, and the level of resistance to aminoglycosides was studied by determination of the MICs (Table 2). The effect of mutational inactivation of plasmid-cloned *aac(2')-Ib* resulted in a decreased level of resistance of *M. smegmatis* mc²155 harboring plasmid pAC63* with respect to the MICs observed for *M. smegmatis* mc²155/pAC63.

Substrate profile of AAC(2') in pAC100. The crude extracts prepared from either *M. smegmatis* mc²155/pAC20 or *M. smegmatis* mc²155/pAC100 were shown to efficiently acetylate gentamicin C1 and C1a, tobramycin, netilmicin, and 6'-*N*-ethylnetilmicin as well as other aminoglycosides. However, 2'-*N*-ethylnetilmicin, kanamycin A, and amikacin were poorly acetylated. Because these aminoglycosides lack the amino group at the 2' position, this confirmed the presence of 2'-*N*-acetyltransferase activity within pAC100. The substrate profile of the crude extract of *M. smegmatis* mc²155/pAC100 is shown in Table 3.

TABLE 3. Substrate profile of the crude extract from *M. smegmatis* mc²155/pAC100

Aminoglycoside	% Acetylation ^a
Gentamicin C1.....	100
Gentamicin C1a.....	90
Tobramycin.....	90
Dibekacin.....	87
Amikacin.....	4
Kanamycin A.....	8
Kanamycin B.....	85
2'- <i>N</i> -Ethylnetilmicin.....	6
6'- <i>N</i> -Ethylnetilmicin.....	61
Netilmicin.....	47
Sisomicin.....	87
5'-Episomicin.....	39

^a Acetylation is expressed relative to that of gentamicin C1, which was defined as 100%. The negative control reaction represented 1.5% acetylation.

Some AAC(2') activity was observed in crude extracts from *M. smegmatis* mc²155. However, the determination of enzymatic activity showed a 20-fold increase of specific activity in the extract from *M. smegmatis* mc²155/pAC100 (45.3 U/μg of protein) with respect to that in the extract from *M. smegmatis* mc²155 (2.19 U/μg of protein).

Analysis of the deduced AAC(2')-Ib protein. The sequences of the AAC(2')-Ia enzyme from *P. stuartii* and the AAC(2')-Ib protein from *M. fortuitum* were compared by using the program Pileup, and the alignment is presented in Fig. 3. The enzymes have a similar size (178 and 195 amino acids, respectively), and the comparison of the deduced protein sequences showed 38% identity and 63% amino acid similarity, indicating that both enzymes belong to the same family of AACs.

Genetic location and distribution of the *aac(2')-Ib* gene between *M. fortuitum* strains and other mycobacteria. A 419-bp *Bam*HI-*Pst*I internal fragment of the *aac(2')-Ib* gene was used as a probe in hybridization experiments with the *M. fortuitum* strains. Dot blot hybridizations with 34 strains of both susceptible and resistant *M. fortuitum* showed that 100% of the strains carried the *aac(2')-Ib* gene (data not shown). Seven of these strains including clinical and environmental isolates were chosen, and their DNAs were digested with *Eco*RI. Southern blot hybridization showed that the seven strains hybridized in a unique band of 10 kb, indicating that the gene was located in the same restriction fragment.

Filters containing DNA from different fast-growing mycobacteria (*M. smegmatis*, *M. chelonae*, and *M. aurum*) were also hybridized, but no hybridization was observed when the filters were washed under high-stringency conditions. However, when the washing conditions were less stringent, all the species hybridized in different bands (data not shown), suggesting that other fast-growing mycobacterial species may have genes homologous to *aac(2')-Ib*.

The findings presented above and the negative results from different attempts to isolate plasmid DNA from *M. fortuitum* FC1K lead us to suspect that the gene *aac(2')-Ib* could be chromosomally encoded.

DISCUSSION

Rapidly growing mycobacteria are naturally resistant to many antibiotics, including aminoglycosides. The presence of AAC enzymes is extensively observed in some fast-growing mycobacteria, with no correlation between resistance levels and the presence of AAC activity (34). Since little is known about the genetic determinants of aminoglycoside resistance in

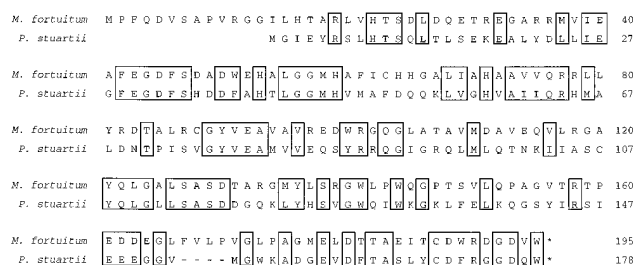


FIG. 3. Comparison of the deduced amino acid sequences of enzymes AAC(2')-Ia (from *P. stuartii*) and AAC(2')-Ib (from *M. fortuitum*). Hyphens represent gaps introduced by the Pileup program to optimize the alignment. Identical or similar amino acids are boxed.

mycobacteria, we decided to study the genes responsible for the AAC activity in *M. fortuitum*.

The substrate profile observed in the crude enzymatic extracts of all the strains of *M. fortuitum* tested was consistent with the presence of an AAC(3) enzyme; i.e., gentamicin, tobramycin, and kanamycins A and B were extensively acetylated (34). This led us to use the probes derived from the *aac(3)-Ia*, *aac(3)-IIIa*, *aac(3)-IVa*, and *aac(3)-VIIa* genes, with no significant results even when the probes were used under low-stringency conditions. The same negative results were obtained in attempts to construct genomic libraries by selecting for gentamicin resistance in *E. coli* or *S. lividans*. Finally, gentamicin-resistant clones of *M. smegmatis* mc²155 were obtained when the strain was transformed with a genomic library of *M. fortuitum*.

We cloned the gene *aac(2')-Ib* from *M. fortuitum* FC1K. This is the structural gene for the AAC(2')-Ib enzyme which confers resistance to gentamicin and other aminoglycosides when it is cloned in *M. smegmatis* mc²155. The deduced sequence of the AAC(2')-Ib enzyme was shown to be 38% identical and 63% similar to the AAC(2')-Ia enzyme from *P. stuartii* [the only AAC(2') described at present]. No significant homology has been found with other AACs, including all of the AAC(3) and AAC(6') proteins. The mycobacterial gene *aac(2')-Ib* is the first gene encoding for AAC activity in members of the order *Actinomycetales* other than the aminoglycoside-producing *Streptomyces* species.

The changes in the MICs observed for *M. smegmatis* harboring plasmids pAC20 or pAC100 with respect to that for the control strain were characteristic of an AAC(2') resistance profile, that is, an increase in the MICs of gentamicin, tobramycin, dibekacin, netilmicin, and 6'-*N*-ethylnetilmicin, whereas there was no increase in the MIC of 2'-*N*-ethylnetilmicin was seen. The MICs of kanamycin A and amikacin were not studied because of the presence of the enzyme APH(3')-II from Tn5 as a selectable marker in those plasmids.

Moreover, the substrate profile of pAC100 showed that gentamicin, tobramycin, dibekacin, netilmicin, 6'-ethylnetilmicin, sisomicin, and 5'-episisomicin were acetylated, whereas kanamycin A, amikacin, and 2'-*N*-ethylnetilmicin were not. Because the latter aminoglycosides lack the 2' amino group, this substrate profile shows that this 2' amino group would be the site of acetylation, confirming the presence of AAC(2') activity. However, because this substrate profile does not explain the acetylation of kanamycin A found in crude extracts of *M. fortuitum* FC1K, we suppose that there is another AAC capable of modifying kanamycin A, such as AAC(3) enzymes. The simultaneous presence of both AAC(2') and AAC(3) enzymes has been described in some *Streptomyces* species (11).

No plasmid could be detected in *M. fortuitum* FC1K even

when other *M. fortuitum* plasmids were successfully isolated in our laboratory (8). These findings and the fact that *aac(2')-I* genes seem to be universally present in all the strains and species of mycobacteria led us to suspect that the gene *aac(2')-Ib* is chromosomally located rather than plasmid-borne. Previously, no relation between the presence of plasmids and the presence of AAC activity in mycobacteria has been described (32). Since *aac(6')-Ic*, the first chromosomally encoded *aac* gene was described in *S. marcescens* (28), other examples have been reported: *aac(2')-Ia* in *P. stuartii* (24), *aac(6')-Ig* in *Acinetobacter haemolyticus* (15), *aac(6')-Ii* in *Enterococcus faecium* (6), *aac(6')-Ij* in *Acinetobacter* sp. 13 (14), and *aac(6')-Ik* in *Acinetobacter* sp. 6 (25). These genes share three main characteristics: they are chromosomally encoded, they are species specific, and they are present in all strains of one bacterial species, which confers taxonomic importance on the chromosomally located *aac* genes.

We have studied the presence of the *aac(2')-Ib* gene in other *M. fortuitum* strains by hybridization. We have demonstrated that the *aac(2')-Ib* gene is universally present in all the *M. fortuitum* strains tested, but its presence is independent of the level of resistance to aminoglycosides. This suggests that the product of this gene could be implicated in functions other than aminoglycoside modification. In general, bacterial acetyltransferases are involved in a variety of cellular processes including acetylation of intermediate metabolites, ribosomal proteins, and cell wall components. Recently, the enzyme AAC(2')-Ia has been shown to contribute to the acetylation of peptidoglycan in *P. stuartii* (18). This strongly supports the hypothesis that aminoglycoside-modifying enzymes may be derived from genes implicated in diverse cellular processes. Other studies (33) have indicated that mycobacterial AACs are inhibited by some amino sugars and derivatives of coenzyme A other than acetyl coenzyme A. These findings indicate that these enzymes are capable of acetylating aminoglycosides and other molecules by using different derivatives of coenzyme A, suggesting that they are probably implicated in other metabolic processes such as biosynthesis of the cell wall. If this hypothesis is confirmed, it would open the possibility of discovery of new molecules that interfere in cell wall synthesis and that could be used as antimycobacterial agents.

Hybridization with *aac(2')-Ib* as a probe under low-stringency conditions showed a signal in other fast-growing mycobacterial strains such as *M. chelonae*, *M. aurum*, and *M. smegmatis* (data not shown). This suggests that genes homologous to *aac(2')-Ib* could be present in other fast-growing species. Indeed, the presence of AAC(2') activity was previously detected in one strain of *M. aurum* (27), and we have also detected this activity in *M. smegmatis* mc²155 in the course of the present work. We propose to extend the study to other fast-growing and slowly growing mycobacterial species and to determine whether these genes could be implicated in functions other than aminoglycoside resistance.

Other characteristics of the chromosomally located AAC genes are that they have unusual transcription signals and are regulated. In this way, genes *aac(2')-Ia* and *aac(6')-Ic* from *P. stuartii* and *S. marcescens*, respectively, have the sequence CTT TTTT at the -35 region (24, 28); further determination of the 5' terminus of the *aac(2')-Ib* mRNA will allow us to observe if such a sequence is also present in *M. fortuitum*. In *P. stuartii*, the expression of the *aac(2')-Ia* gene is controlled at the transcriptional level by several *trans*-acting regulatory factors coded by the *aarA*, *aarB*, and *aarC* genes (23, 24) and a transcriptional activator coded by *aarP* which increased the level of *aac(2')-Ia* expression (18). Such a complex regulatory process may indicate that this gene has an important role in secondary

metabolism rather than aminoglycoside modification. Single-step mutants of *P. stuartii* showing increased levels of resistance to aminoglycosides provided the key to discovering the regulatory pathway of the *aac(2')-Ia* gene (24). We have also detected gentamicin-resistant mutants of *M. smegmatis* in the course of the cloning of *aac(2')-Ib*, mutants that will be further studied to determine whether the resistance is due to an increased level of expression of the *aac(2')-Ib* gene. Further studies are being conducted.

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