Determination of a 15437 bp nucleotide sequence around the *inhA* gene of *Mycobacterium avium* and similarity analysis of the products of putative ORFs

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A 15437 bp region encompassing the *inhA* locus from the *Mycobacterium avium* chromosome was cloned and sequenced. From the sequencing data generated and the results of homology searches, the primary structure of this region was determined. This region contains four known genes (*acnA*, *fabG*, *inhA* and *hemH*) and two genes, *invA* and *invB*, whose products display homology with p60 invasion protein of *Listeria monocytogenes*. Six proteins encoded by putative ORFs contained an RGD motif (often involved in binding to macrophage integrins), while ORF1 and MoxR are probably transcriptional regulators. The rest of the putative products encoded by ORFs in the sequenced region showed little homology with the proteins contained in the databases and were considered to be unknown proteins.

Keywords: *inv* genes, *inhA* gene, *Mycobacterium avium*, nucleotide sequence

INTRODUCTION

Mycobacteria, members of which cause tuberculosis and leprosy, produce cell walls of unusually low permeability which contribute to their resistance to therapeutic agents. Their cell walls contain large amounts of C₆₀–C₉₀ fatty acids, mycolic acids, that are covalently linked to arabinogalactan. Differences in mycolic acid structure may affect the fluidity and permeability of the bilayer and may explain the different sensitivity levels of various mycobacterial species to lipophilic inhibitors (Liu *et al.*, 1996). There are several indications that some existing antimycobacterial drugs may indeed act on the cell wall biosynthetic pathways (Blanchard, 1996). Identification of the precise targets of such drugs may allow design of novel inhibitors of the same enzyme or of related steps in the same pathway.

Several mechanisms seem to be involved in isoniazid (INH) resistance in *Mycobacterium tuberculosis* (Deretic *et al.*, 1996). The simplest hypothesis is that the

drug is converted inside the bacterium to an activated form by the catalase–peroxidase enzyme, encoded by the *katG* gene (Zhang *et al.*, 1993), and that the activated drug then inhibits the product of the *inhA* gene, an enzyme probably involved in mycolic acid biosynthesis (Banerjee *et al.*, 1994). *InhA* has been shown to catalyse the β -NADH-specific reduction of 2-*trans*-enoyl-acyl carrier protein, an essential step in fatty acid elongation (Dessen *et al.*, 1995). The *M. tuberculosis inhA* gene appears to be in an operon with the gene encoding the 29 kDa ORF that exhibits sequence similarity to several other proteins involved in fatty acid biosynthesis, such as the 3-ketoacyl-acyl carrier protein reductase (*fabG* gene product) from *Escherichia coli* (Banerjee *et al.*, 1994).

The *Mycobacterium avium* complex is an intracellular pathogen that predominantly infects and multiplies within macrophages. *M. avium* infections are frequently present in patients with AIDS (Inderlied *et al.*, 1993). Treatment of infections caused by *M. avium* still remains a challenge since these organisms are resistant to the majority of antituberculous drugs, presumably because of their impermeability to these agents. The bacterial cell envelope architecture could act as a barrier to some of the drugs (Brennan & Nikaido, 1995). While the complex cell wall structure of mycobacteria prob-

Abbreviation: INH, isoniazid.

The GenBank accession numbers for the sequences reported in this paper are AF002133 for *Mycobacterium avium* and AF006054 for *Mycobacterium tuberculosis*.

ably confers the permeability barrier that underlies their resistance to many existing antibacterial agents, the same unique structure contains a series of potential targets for novel *Mycobacterium*-specific inhibitors. The mycolic acids are unique to the mycobacteria and, again, it can be envisaged that their synthesis and assembly into the cell wall entail a series of enzymes, each representing a potentially attractive target for antibacterial action.

Since the *inhA* gene seems to be directly involved in mycolic acid biosynthesis, we have sequenced and analysed 15437 bp around this region to establish if other related genes are present in *M. avium*.

METHODS

Isolation of the *inhA* locus from a λ GEM-11 library of *M. avium*. The *Mycobacterium smegmatis inhA* gene (kindly provided by Professor W. R. Jacobs, Albert Einstein College of Medicine, NY, USA) was used as probe to screen a λ GEM-11 library of *M. avium* GIR10 (Milano *et al.*, 1996). Several positive phages were identified and one of them, λ GEM-33, was chosen to map the *M. avium inhA* gene. DNA fragments from λ GEM-33 were subcloned into pGEM-4Z (Promega) for sequencing.

Procedures such as plaque hybridization and synthesis of radioactive DNA probes with random hexamer primers were carried out using standard protocols (Sambrook *et al.*, 1989).

Genomic and λ GEM-33 DNAs were digested with appropriate restriction endonucleases, fractionated on a 1% agarose gel and transferred to a nylon membrane (Schleicher & Schuell) according to the manufacturer's instructions. Hybridization was performed at 42 °C with 50% formamide; the blot was washed at 60 °C in 1 × SSC/0.1% SDS.

To isolate and clone the corresponding *inv* genes from *M. tuberculosis*, we used the homologous PCR product as a probe to screen an *M. tuberculosis* cosmid library (kindly provided by Dr S. Cole, Institut Pasteur, Paris, France). Several positive cosmids were isolated and the *M. tuberculosis inv* genes were cloned and sequenced by standard procedures.

DNA sequencing and analysis. The *M. avium* DNA fragment from λ GEM-33 was digested with several restriction enzymes and the resulting fragments were subcloned into pGEM-4Z. Sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977), using the Pharmacia T7 Polymerase Sequencing kit according to the supplier's instructions with [³⁵S]dATP α S. The sequence was determined on both strands and all restriction sites used to subclone into pGEM-4Z were overlapped. The subclones were sequenced using the SP6 and T7 promoter primers for pGEM-4Z as well as synthetic oligonucleotide primers designed from the resulting sequence.

DNA and protein sequences were compared with non-redundant databases at the National Center for Biotechnology Information (NCBI), using the BLAST programs (Altschul *et al.*, 1990, 1994). The putative ORFs were identified both with the NCBI ORF-FINDER and with the PC/GENE programs. Putative ORFs in the region were sought in all six possible translation frames. Further analysis was performed using the PROSITE catalogue (Bairoch, 1992). Calculations of the isoelectric point and of the molecular mass were determined by COMPUTE pI/Mw (Bjellqvist *et al.*, 1993, 1994). Multiple

alignment was performed with the CLUSTAL W program (Thompson *et al.*, 1994).

PCR procedures. DNA for PCR analysis, from different mycobacterial species, was extracted by the following procedure. A loopful of organisms from a colony was suspended in 1 ml H₂O and boiled for 10 min. Samples (10 μ l) were then used directly in PCR reactions using the primers 5'-GGGGGTGCCGTACTCGTGGGG-3' (upper) and 5'-ACCCGCCCGGGCCGTAGAAG-3' (lower).

RESULTS AND DISCUSSION

We used the *M. smegmatis inhA* gene (Banerjee *et al.*, 1994) as a probe to isolate the corresponding gene from a λ GEM-11 library of *M. avium* (Milano *et al.*, 1996). Several positive recombinants were selected and one of them (λ GEM-33) was further studied. This recombinant phage carried a total insert of 15437 bp and its physical map is shown in Fig. 1. Hybridization experiments revealed a fragment whose size was consistent with that predicted by the DNA sequence of the λ -insert (data not shown).

ORFs present in the resultant 15437 bp segment, which has a G + C content of 69 mol%, typical of *M. avium*, were identified on the basis of their codon usage and database search similarities (Tables 1 and 2). The end points of the ORFs in the sequence, the size of the protein encoded, isoelectric point, putative Shine-Dalgarno sequence and initiation codon are shown in Table 1. Many of the genes appear to comprise independent monocistronic transcriptional units although there are some discernible operons. Similarities of predicted ORF products with database protein sequences and matches to PROSITE motifs (Table 2) led to the identification of many of the genes and their biological functions.

Of our sequence, 13 170 bp in total was found to encode protein. The remaining 2267 bp seems to be non-coding. The largest non-coding stretch, 1506 bp (from nt 3167 to nt 4673), was without apparent structural features, as well as the 644 bp second non-coding stretch.

M. avium inhA gene

From positions 10566 to 11333 and from 11368 to 12174, we found, respectively, the *fabG* and *inhA* genes, as previously identified in *M. tuberculosis* (Banerjee *et al.*, 1994). The predicted InhA protein of *M. avium* shows strong sequence similarity (85.5% identity in a 269-residue overlap) to the corresponding protein of *M. tuberculosis*. A serine-94 → alanine-94 mutation of the *inhA* gene of *M. smegmatis* and of *Mycobacterium bovis* has been shown to cause resistance to INH and ethionamide (Banerjee *et al.*, 1994) by decreasing the affinity of the mutant protein for NADH (Dessen *et al.*, 1995). The structure of the NADH binding site of InhA suggests that recognition of NADH is mediated by interactions with an array of polar amino acids and backbone atoms (Dessen *et al.*, 1995). The residues which are involved in the NADH-binding site of InhA

Fig. 1. Restriction map and ORF organization of the *M. avium* chromosome around the *inhA* gene. B, *Bam*HI restriction sites; S, *Sac*I restriction sites; ■, RGD motif.

Table 1. Putative ORFs and characteristics of their products in the 15437 bp sequence around the *inhA* gene of the *M. avium* chromosome

ORF	End points (nt)	Molecular mass (kDa)	pI	SD* consensus sequence (upper case) and initiation codon (bold)
<i>orf1</i>	246 < 1	Partial	Partial	AtGcccAGctAGATG
<i>orf2</i>	3167 < 282	104.0	5.2	GAccGccGAcGtaATG
<i>orf4</i>	4673 > 5494	28.8	9.6	AGGAcGccGcGGTG
<i>orf5</i>	5520 > 6254	25.4	9.6	AAcGAGGAGccATG
<i>orf6</i>	6376 > 7305	34.2	10.9	GGAGGGttAtcGATG
<i>orf8</i>	7949 > 8458	18.6	10.7	AAcGGcGccAccATG
<i>orf9</i>	8466 > 9473	36.0	9.4	GActcGAAAtATG
<i>orf10</i>	10626 < 9586	39.5	10.3	GGGAGAcGAATG
<i>orf11</i>	9733 > 10569	30.6	11.5	AGcGGctcGcccGATG
<i>orf12</i>	11414 < 10509	30.8	5.4	GAcGAGtcGGtGATG
<i>orf13</i>	10566 > 11333	26.7	5.6	AGGttGAcGGGGTG
<i>orf14</i>	11368 > 12174	28.5	5.0	AAAGGAcGGAcATG
<i>orf15</i>	12182 > 13189	29.8	11.8	AGAcGGGcAATG
<i>orf16</i>	13193 < 14155	33.4	5.5	cAAGAAAtAcGcGTG
<i>orf17</i>	13211 > 14128	33.7	11.9	AGGcGGAATG
<i>orf18</i>	14171 > 14608	15.6	5.6	ttcttgccgcATG
<i>orf19</i>	14639 > 15437	Partial	Partial	AAAGGAAttccAATG

* SD, Shine–Dalgarno.

from *M. tuberculosis* wild-type are unchanged in the *M. avium* *InhA* sequence; this finding rules out a possible involvement of *M. avium* *InhA* in the natural resistance of the micro-organism to INH.

In *M. avium* and in *M. tuberculosis* DNA, the non-coding region between the two ORFs is short and may lack a promoter; consequently, the two genes may be organized in an operon. Immediately downstream of the *inhA* gene (from nt 12182 to nt 13189) there is *orf15*, whose gene product shows similarity with several ferroxidase enzymes. The last step in haem synthesis is the insertion of iron into the ring of protoporphyrin IX. The enzyme which catalyses this reaction is a ferroxidase, encoded by the *Rhodobacter capsulatus* *hemH* gene (Kanazireva & Biel, 1995). The degree of sequence homology of *M. avium* ORF15 compared with human ferroxidase (Crouse *et al.*, 1996) is 34% identity in a 132-residue overlap. The same gene organization is present in *M. tuberculosis* (accession no.

gnl/Z79701) and the *fabG*–*inhA*–*hemH* genes could be organized in an operon in both micro-organisms (Fig. 2).

invA and *invB* genes

Little is known about the bacterial factors that enable pathogenic mycobacteria to survive and multiply within the macrophages of the infected host. *M. tuberculosis* bacilli are thought to enter the macrophage via specific binding to complement receptors and the macrophage mannose receptor (Fenton & Vermeulen, 1996). The neural tropism of *Mycobacterium leprae* has been ascribed to the specific binding of *M. leprae* to the laminin- α_2 (LN- α_2) chain on Schwann cell axon units. It has been suggested that *M. leprae* interacts with cells by binding to β_4 integrin via an LN- α_2 G bridge (Rambukkana *et al.*, 1997). Recently, it has been demonstrated that the *M. avium* 68 kDa protein mediates attachment of *M. avium* to macrophages, by interacting with $\alpha_v\beta_3$ on these cells (Hayashi *et al.*, 1997).

Table 2. Similarity of the putative products of ORFs identified on λ GEM-33 *M. avium* insert

ORF	Length (aa)	Sequence similarity and/or PROSITE match	BLAST score (P-value)*
<i>orf1</i>	82	AcrR from <i>E. coli</i> (sp/P34000)	2.4e-08
<i>acn</i>	961	Aconitase from: <i>E. coli</i> (sp/P25516)	0.0
		<i>L. pneumophila</i> (sp/P37032)	0.0
		<i>Brad. japonicum</i> (U56817)	0.0
		<i>Bac. subtilis</i> (Z73234)	0.0
<i>invA</i>	273	Hypothetical invasion protein similar to: <i>M. tuberculosis</i> ORF MTCY336.37 (gb/Z95586)	8.1e-53
		<i>M. leprae</i> Lepb1170_F3_153 (U00010)	2.2e-43
		<i>Listeria ivanovii</i> protein p60 (sp/Q01837)	9.1e-17
		PS00016: RGD cell attachment sequence	
<i>invB</i>	244	Hypothetical invasion protein similar to: <i>M. tuberculosis</i> ORF MTCY336.37 (gb/Z95586)	6.4e-48
		<i>M. leprae</i> Lepb1170_F3_153 (U00010)	1.4e-45
		<i>Listeria ivanovii</i> protein p60 (sp/Q01837)	4.3e-15
		PS00016: RGD cell attachment sequence	
<i>moxR</i>	309	Hypothetical transcriptional regulator similar to: <i>Bor. burgdorferi</i> <i>moxR</i> gene product (X96434)	3.6e-64
		<i>Bac. subtilis</i> YeaC (U51115)	3.1e-61
		<i>Synechocystis</i> sp. MoxR (D64003)	1.6e-59
<i>orf8</i>	169	Unknown High similarity to <i>M. tuberculosis</i> partial reading frame MTCY227.01 (Z79701)	1.2e-49
		PS00016: RGD cell attachment sequence	
<i>orf9</i>	335	Unknown High similarity to <i>M. tuberculosis</i> MTCY227.02 (Z79701)	1.5e-207
<i>orf10</i>	346	Unknown High similarity to <i>M. tuberculosis</i> MTCY227.03 (Z79701)	5.3e-151
<i>orf11</i>	278	Unknown PS00016: three RGD cell attachment sequences	
<i>orf12</i>	301	Unknown	
<i>fabG</i>	255	Ketoacyl-reductase High similarity to <i>M. tuberculosis</i> FabG (Z79701)	1.5e-50
<i>inhA</i>	268	Enoyl-reductase High similarity to <i>M. tuberculosis</i> InhA (Z79701)	4.0e-154
<i>hemH</i>	335	Gene encoding ferrochelatase High similarity to corresponding protein of <i>M. tuberculosis</i> (Z79701)	9.2e-93
		PS00016: RGD cell attachment sequence	
<i>orf16</i>	321	Unknown High similarity to <i>M. tuberculosis</i> MTCY227.07 (Z79701)	1.3e-154
<i>orf17</i>	305	Unknown PS00016: two RGD cell attachment sequences	
<i>orf18</i>	145	Unknown Similarity to <i>M. tuberculosis</i> MTCY227.08 (Z79701)	1.1e-67
<i>orf19</i>	266	Integral membrane protein High similarity to <i>M. tuberculosis</i> MTCY227.09 (Z79701)	2.6e-134
		Similarity to integral membrane protein from: <i>Methanococcus jannaschii</i> (U67526)	4.9e-50
		<i>Synechocystis</i> sp. (D90899)	6.3e-45

* The P-value, calculated with the BLAST program, represents the probability that two proteins are related. The lower the P-value, the greater the degree of similarity.

Fig. 2. Physical organization of *fabG*–*inhA*–*hemH* in *M. avium* and *M. tuberculosis*.

orf4 (from nt 4673 to nt 5494) and *orf5* (from nt 5520 to nt 6254) show a low but detectable level of homology with p60, a protein that is involved in the uptake of *Listeria monocytogenes* by non-professional phagocytic cells (Bubert *et al.*, 1992), and that is probably essential for survival inside the phagosome (Bhunia, 1997). Further, p60 is a murein hydrolase that is secreted into the host cell cytosol, where it is degraded by proteasomes (Sijts *et al.*, 1997). The P-values for ORF4 and ORF5 compared with the p60 protein were 9.1×10^{-17} and 4.3×10^{-15} , respectively. The highest score, 8.1×10^{-53} for ORF4 and 6.4×10^{-48} for ORF5, was observed with the ORF MTCY336.37 from *M. tuberculosis* (accession no. gb/Z95586). Similar scores, 2.2×10^{-43} for ORF4 and 1.4×10^{-45} for ORF5, were with Lepb1170_F3_153 from *M. leprae* (accession no. gi/U00010). As *M. avium* ORF4 and ORF5 show homology with p60, have an RGD motif and are 59.4% identical in a 219-residue overlap, we decided to name the corresponding genes *invA* and *invB*, respectively. These genes appear to be in an operon as they are separated by only 26 bp.

To evaluate the presence of *inv* genes within the mycobacterial genus, we used PCR, utilizing the upper and lower primers (see Methods) from *M. avium invB*. As shown in Fig. 3, a band of amplified DNA, with the appropriate size, appeared in all mycobacteria strains tested. This result is not surprising since, for example, the *iap* gene (encoding p60) has been found in several tested *Listeria* spp. (pathogenic and non-pathogenic) (Kohler *et al.*, 1990).

The *M. tuberculosis* PCR product was used as a probe to screen a corresponding cosmid library. The isolated *M. tuberculosis* clone was mapped and 2360 bp was sequenced. Sequence analysis revealed the presence of two *M. tuberculosis inv* genes and a physical organization very similar to that of *M. avium* (data not shown).

Fig. 4 shows the alignment of amino acid sequences of *M. avium* InvA, *M. avium* InvB, *M. tuberculosis* InvA, *M. tuberculosis* InvB, *M. leprae* Inv, *M. tuberculosis* ORF MTCY336.37 and C-terminal *L. monocytogenes* p60. As shown in Fig. 4, several amino acid blocks are conserved between Inv proteins and p60 at the C-terminal domain. It has been demonstrated that the p60 cysteine residue at position 39 is essential for the hydrolytic activity of the protein (Wuenschel *et al.*, 1993). The same residue is conserved in *M. avium* and *M. tuberculosis* Inv proteins. As shown in Fig. 4, five proteins contain an RGD motif, which is common to many extracellular proteins and which is thought to play a key role in cell adhesion (Rouslahti & Pierschbacher, 1986, 1987). RGD sequences have also been identified in bacterial proteins and have been shown to interact with host receptors (Frankel *et al.*, 1996; Everest *et al.*, 1996). However, non-RGD-mediated interactions between bacterial ligands and their host cell integrin receptors have also been reported (Leong *et al.*, 1990). Whether the *M. avium* and *M. tuberculosis* Inv proteins are really involved in adhesion–invasion processes remains to be determined.

Fig. 3. Detection by PCR of the *inv* genes from mycobacterial species. Lanes: 1, *M. simiae*; 2, *M. chelonae*; 3, *M. goodii*; 4, *M. marinum*; 5, *M. bovis* BCG; 6, *M. flavescens*; 7, *M. fortuitum*; 8, *M. kansasii*; 9, *M. xenopi*; 10, *M. terrae*; 11, *M. smegmatis*; 12, *M. abscessus*; 13, *M. avium*; and 14, *M. tuberculosis* H37Rv. The fainter PCR amplification product observed in *M. bovis* BCG was due to the quality of the sample, and was brighter in other experiments.

tuberculosis genes are homologues. This would also explain the lack of a downstream homologue of *M. leprae* Lepb1170_F3_153. A striking feature of ORF MTCY336.37 is a domain consisting of eight QQAPV repeats in the C-terminal region.

We also analysed *M. avium* InvA and InvB for potential membrane proteins. For this purpose, the amino acid sequences were scanned with TMbase (Hofmann & Stoffel, 1993). The results indicated two strong trans-membrane helices for InvA and one strong trans-membrane helix for InvB (data not shown).

There is an immediate need to understand the molecular basis of virulence and the pathogenesis of *M. tuberculosis* before the current molecular biological and immunological tools can be used in the prevention and control of the disease. Pathogens entering the human body must quickly adapt to changes in temperature, pH, osmotic strength, oxygen availability and nutrient concentration. Successful pathogens have evolved the ability to regulate expression of virulence genes.

invA/invB gene expression has been exploited in *M. tuberculosis* extracellular and intracellular growth. From preliminary data, it seems that *M. tuberculosis invB* expression is induced only in response to growth within the macrophage (unpublished data). Other experiments are in progress to evaluate the function of InvA and InvB in mycobacterial pathogenesis.

Downstream of *M. avium* *orf5* (*invA*) is *orf6* (from nt 6376 to nt 7305), whose product showed high similarity to MoxR of *Borrelia burgdorferi* (P-value 3.6×10^{-64} ; accession no. gi/X96434), MoxR of *Synechocystis* sp. (P-value 1.6×10^{-59} ; accession no. gi/D64003) and YeaC of *Bacillus subtilis* (P-value 3.1×10^{-61} ; accession no. gi/U51115). MoxR seems to be involved in the regulation of formation of active methanol dehydrogenase in *Paracoccus denitrificans* (van Spanning *et al.*, 1991). Another transcriptional regulator could be ORF1, whose 82 amino acids showed similarity (35.6% identity in a 59-residue overlap) to *E. coli* AcrR, a repressor that plays a modulating role in the regulation of *acrAB* genes of *E. coli* (Ma *et al.*, 1996).

Aconitase

Aconitases are monomeric proteins containing single labile [4Fe-4S] clusters that possess a versatile molecular structure adapted to catalyse reactions with citrate, isopropylmalate and homocitrate and to function as iron-responsive regulators of mRNA translation or stability (Klausner & Rouault, 1993; Rouault & Klausner, 1996; Hentze & Kuhn, 1996). Crystallographic studies with porcine-heart mitochondrial aconitase have revealed the presence of three structural domains (1, 2 and 3) tightly packed around the [Fe-S] cluster and a fourth domain (4) that is connected by a long linker peptide in such a way as to create a deep active-site cleft (Robbins & Stout, 1989). This arrangement provides the

structural prototype for the aconitase family (Gruer *et al.*, 1997a).

Only a few bacterial aconitases have been studied so far. Two distinct aconitases, AcnA and AcnB, were discovered in *E. coli*. AcnB is the major citric acid cycle enzyme, subjected to anaerobic and catabolite repression, whereas AcnA is induced by redox stress and by the presence of iron (Prodromou *et al.*, 1991; Bradbury *et al.*, 1996). Aconitase has also been characterized in *Bac. subtilis* (Dingman & Sonenshein, 1987), *Legionella pneumophila* (Mengaud & Horwitz, 1993) and *Bradyrhizobium japonicum* (Thony-Meyer & Kunzler, 1996). *E. coli*, *L. pneumophila* and *Bac. subtilis* AcnA proteins, together with the iron-regulatory proteins and plant aconitases, form an iron-regulatory protein/AcnA subfamily whose members are more closely related to each other than to any other members of the aconitase protein family (Gruer *et al.*, 1997b).

M. avium *orf2* starts at position 3167 (complementary strand) with an ATG codon that is preceded by a Shine-Dalgarno-like sequence (5'-CGACG-3') at a distance of 7 nt and ends at position 282 with a TGA stop codon. The derived polypeptide has a predicted molecular mass of 104 kDa and an isoelectric point of 5.27. The deduced amino acid sequence shows high similarity with *E. coli* aconitase AcnA (80% similarity, 54.1% identity) as well as with the bacterial aconitases from *L. pneumophila*, *Brad. japonicum* and *Bac. subtilis*. The similarity between the *M. avium* and *Cucurbita maxima* (pumpkin seed) aconitases (Hayashi *et al.*, 1995) was in the range of 76.7% (47.6% identity). By contrast, *M. avium* aconitase is less related (34.6% identity) to porcine mitochondrial aconitase (Zheng *et al.*, 1990). A good degree of similarity was also observed with eukaryotic iron-response-element-binding proteins (P-value 5e-305).

It is noteworthy that the cytoplasmic aconitase holoenzyme containing the [Fe-S] cluster is in equilibrium with its apoprotein, the iron-response-element-binding protein, which has a regulatory function in iron transport and storage. Hence the function of this protein depends on the availability of intracellular iron for the formation of the [Fe-S] cofactor (Klausner & Rouault, 1993). Crystallographic structure analysis and mutational analysis have led to the identification of 23 residues contributing to the active site (Lauble *et al.*, 1992; Zheng *et al.*, 1992). The 23 residues, which are involved in substrate recognition, [Fe-S] cluster ligation and interaction, catalysis or hydrogen binding that support the active-site side chains are also present in the *M. avium* AcnA sequence (data not shown).

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