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Fast-growing, non-infectious and intracellularly surviving drug-resistant *Mycobacterium aurum*: a model for high-throughput antituberculosis drug screening

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***Objectives*: Enoyl acyl-carrier-protein reductase (InhA), the primary endogenous target for isoniazid and ethionamide, is crucial to type-II fatty acid biosynthesis (FAS-II). The objectives of this study were first to generate InhA mutants of *Mycobacterium aurum*, secondly to characterize InhA-mediated isoniazid and ethionamide resistance mechanisms across those mutants and finally to investigate the interaction of InhA with enzymes in the FAS-II pathway in *M. aurum*.**

***Methods*: Spontaneous mutants were generated by isoniazid overdose and limited broth dilution, while for genetically modified mutants sense–antisense DNA technology was used. Southern hybridization and immunoprecipitation were both used to identify the InhA homologue in *M. aurum*. The latter method was further used to compare the level of InhA expression in *M. aurum* with that in corresponding mutants. Isoniazid/ethionamide susceptibility modulation was examined *in vitro* and *ex vivo* using a resazurin assay as well as by cfu counting. In addition, circular dichroism and the bacterial two-hybrid system were exploited to investigate the interaction of InhA with other enzymes of the FAS-II pathway.**

***Results*: A *Mycobacterium tuberculosis* InhA homologue was detected in *M. aurum*. Susceptibility to isoniazid/ethionamide was significantly altered in genetically modified mutants and simultaneously InhA was overexpressed in both spontaneous and genetically modified mutants. InhA interacts with other FAS-II enzymes of *M. aurum in vivo*.**

***Conclusion*: Close resemblance of isoniazid/ethionamide action on InhA between *M. tuberculosis* and *M. aurum* further supports the use of fast-growing and intracellularly surviving drug-resistant *M. aurum* to substitute for highly virulent, extremely slow-growing *M. tuberculosis* strains in the early stage of antituberculosis inhibitor screening.**

Keywords: antibacterial drug screening, drug resistance, enoyl acyl-carrier-protein reductase, protein–protein interaction, surrogate

Introduction

The inexorable rise of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) has raised serious public health issues for TB control programmes.[1] The emergence of clinical strains of *Mycobacterium tuberculosis* resistant to effective and widely used drugs, including isoniazid, is extremely concerning. Despite the use of isoniazid for several decades, the molecular basis for its bactericidal action and the mechanisms by which isoniazid resistance evolves in *M. tuberculosis* have only recently been explored.[2] Isoniazid is a prodrug which on activation through the catalase peroxidase

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enzyme encoded by the *katG* gene makes an adduct with NAD(H) to inhibit the enoyl acyl-carrier-protein reductase (InhA) enzyme.[3,4] Although isoniazid seems to inhibit more than one target, the primary target of isoniazid in *M. tuberculosis* has been a debatable issue,[5] until it was established that the InhA enzyme encoded by the *inhA* gene is the primary target of isoniazid.[6,7] InhA was also found as the primary target of another important anti-TB drug ethionamide.[7] This essential enzyme is required for the elongation of acyl fatty acid precursors of the unique cell wall component mycolic acids through the type-II fatty acid biosynthesis (FAS-II) pathway.[8] The enzymes of the FAS-II pathway have been found to be participating through protein–protein interactions, not only to communicate information in their own pathway but also for shuttling the phthiocerol product from phthiocerol dimycocerosate (PDIM) into the FAS-II pathway.[9,10] Moreover, rapid advances in functional genomics involved in identification of non-covalent associations of multienzyme complexes have opened up a strategy to target protein assemblies. Thus inhibitors of protein–protein interactions of the interacting network in the FAS pathway would provide an effective input towards combating the complex resistance mechanisms of these cell wall-inhibiting drugs.

The quest for exploring novel anti-TB chemotherapeutics from the plethora of available inhibitors against MDR and XDR *M. tuberculosis* is severely impeded by the slow growth of this organism and the need to work in a sophisticated and highly stringent biosafety environment that poses considerable obstacles, such as complex handling, expensive set-up and special training requirements. In order to alleviate these critical issues, non-pathogenic fast-growing mycobacteria have been introduced as test organisms in drug screening processes.[11,12] Among all of the non-pathogenic fast-growing mycobacteria, *Mycobacterium aurum* appeared to be closest to *M. tuberculosis* in terms of mycolate components due to the presence of keto-mycolic acid and a cyclopropane ring in both apolar α-mycolic acids and oxygenated mycolic acids.[13,14] Since the structural similarities in mycolates are responsible for the permeability of the cell envelope to antimicrobials, there is a high level of resemblance in the susceptibility profile of cell wall inhibitors in *M. aurum* and *M. tuberculosis*, subsequently recommending its use for the search for new inhibitors.[15,16] The ability to survive in an intracellular environment similar to *M. tuberculosis* is another advantage to utilization of *M. aurum* for the second step of drug screening.[17,18] In addition the use of recombinant *M. aurum* expressing green fluorescent protein, β-galactosidase and luciferase has contributed to rapid screening of anti-TB compounds *in vitro* and in infected macrophages.[18–20] Recently, disruption of the FAS-II pathway through antibiotic stress to the *kas* operon promoter in association with the *lacZ* reporter gene has been addressed as a mechanism-based drug screening system in *M. aurum*.[21]

M. aurum, like *M. tuberculosis*, is highly susceptible to isoniazid; however, in *M. aurum* InhA as a drug target has not yet been explored. Certain evidence, such as lack of *ahpC* expression in *M. aurum*, absence of catalase peroxidase in isoniazid-resistant *M. aurum* and inhibition of mycolic acid synthesis in isoniazid-treated *M. aurum*, suggested that a possible similar mechanism of isoniazid resistance exists in *M. aurum* and *M. tuberculosis*.[22–25]

In this study, we have investigated the role of the primary target *inhA* on isoniazid and ethionamide resistance in *M. aurum* to establish a surrogate for drug-resistant *M. tuberculosis*. Additionally, in order to strengthen the justification for the use of isoniazid/ethionamide-resistant *M. aurum* as a surrogate host for high-throughput drug screening purposes, we have addressed the functional aspects of the InhA enzyme through *in vitro* and *in vivo* interactions with other FAS-II enzymes of *M. aurum*. The results of our study have characterized drug-resistant *M. aurum* strains as a convenient model for *in vitro* and *ex vivo* screening of potential anti-TB inhibitors.

Materials and methods

Bacterial strains, plasmids and growth media

The *M. aurum* A^+ strain was obtained from the mycobacterial repository of the National JALMA Institute for Leprosy and Other Mycobacterial Diseases, Agra, India. The strain was grown in Sauton's medium supplemented with 0.1% Tween-80 and 0.5% glycerol, and plated on nutrient agar with 0.1% Tween-80 (NAT). The *Escherichia coli* strains were grown in Luria–Bertani (LB) broth and LB agar. Kanamycin, ampicillin, isoniazid, ethionamide, gentamicin and isopropyl-β-D-thiogalactopyranoside (IPTG) were prepared in aqueous solution, while 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and *O*-nitrophenyl-β-D-galactopyranoside (ONPG) were prepared in dimethyl formamide. All chemicals were purchased from Sigma. Vectors pTZ57R/T (MBI Fermentas) and pTriEx4 (Novagen) were used for cloning and expression of genes in *E. coli*, and pMV261 in mycobacteria.[26]

Cloning, heterologous expression and purification

The *inhA* (*Rv1484*) gene of *M. tuberculosis* H37Rv,[27] and *fabD*, *kasA*, *kasB*, *acpM* and *accD6* of *M. aurum* (accession no. DQ268649) were PCR amplified from their respective genomic DNAs. Primers used in this study are listed in Table 1. PCR was carried out using Taq DNA polymerase (Invitrogen) with standard cycling conditions. The amplified amplicons were cloned in pTZ57R/T TA cloning vector and transformed into *E. coli* DH5α (Novagen). Transformants containing inserts were selected as blue colonies on LB agar containing X-gal and ampicillin. The clones were confirmed by nucleotide sequencing.

For overexpression in *E. coli*, genes were excised from recombinant plasmids of mt*inhA*, ma*fabD*, ma*acpM* and ma*kasA* using BamHI/EcoRI, and in the case of ma*kasB* and ma*accD6* using BamHI/ClaI (the prefixes 'mt' and 'ma' denote the source of the gene being *M. tuberculosis* or *M. aurum*, respectively), which was then followed by cloning of these genes in pTriEx4. These recombinant plasmids were transformed into *E. coli* BL21 (DE3) Tuner (Novagen) cells. Cells harbouring these constructs were grown at 37°C to an optical density (OD) of 0.4 at 600 nm in LB broth containing ampicillin, induced by 1 mM IPTG, with further incubation at 30°C for 4 h for mtInhA, maFabD, maKasA and maKasB and at 16°C for maAcpM. Cells were harvested and the pellet was suspended in buffer A (20 mM Tris pH 7.5, 500 mM NaCl). The cells were then sonicated and, after removal of debris, the supernatant containing soluble proteins was applied to a column packed with Ni^{2+}-NTA resin (Qiagen) for purification. For insoluble proteins (maKasA and maKasB), extraction was with 6 M urea. His-tag recombinant proteins were eluted with buffer A containing a linear range of 20–500 mM imidazole. Fractions were identified by SDS–PAGE, pooled and dialysed against 20 mM sodium phosphate buffer with 10% glycerol. Purified proteins were stored at −80°C.

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Table 1. Primers used in this work

Designation	Oligonucleotide sequence[a]	Restriction site
inhA F	CGGGATCCCATGACAGGACTGCTGGACGGC	BamHI
inhA R	CGGAATTCTAGAGCAATTGGGTGTGCG	EcoRI
fabD F	CGGGATCCCTTAATCGCACCCGGACAGG	BamHI
fabD R	CGGAATTCAGAGCTCGGCGAGCGTG	EcoRI
acpM F	CGGGATCCCGTGGCCGCCAGCCAGCAG	BamHI
acpM R	CGGAATTCTCATTCCGCGAACTTCTCGC	EcoRI
kasA F	CGGGATCCCATGAGCCGGCCTTCCACTG	BamHI
kasA R	CGGAATTCTTAATAGCGACCGAACGCCAG	EcoRI
kasB F	CGGGATCCCATGGCAGGGGTCAAGGGAC	BamHI
kasB R	CCATCGATTCAGTACTTGCCGAAGGCGA	ClaI
accD6 F	CGGGATCCCATGACAATCATGGCGCCCGA	BamHI
accD6 R	CCATCGATCTACAGCGGGATGTTCTTGTG	ClaI

[a]Restriction enzyme recognition sites are underlined.

For bacterial two-hybrid (BTH) studies, the mt*inhA* gene was subcloned at the BamHI site in vector pKT25 of the BTH system, while ma*fabD*, ma*acpM*, ma*kasA*, ma*kasB* and ma*accD6* genes were subcloned in vector pUT18C at BamHI/EcoRI for the first three genes and at BamHI/ClaI for the last two. The *fabD* gene of *M. tuberculosis* was amplified, cloned in pTZ57R/T and then subcloned in pUT18C at BamHI/EcoRI restriction sites.

Detection of inhA using Southern hybridization

Genomic DNA from *M. aurum* and *M. tuberculosis* was digested with the restriction enzyme SalI, electrophoresed through 0.8% agarose in TAE buffer and transferred on to a nitrocellulose membrane overnight by capillary transfer.[28] After cross-linking the DNA to the membrane, Southern hybridization was carried out with the digoxigenin-labelled mt*inhA* gene as a probe. The probe was labelled and detected according to the manufacturer's instructions.

Development of polyclonal antibody against mtInhA

A rabbit was immunized subcutaneously with 200 μg of mtInhA recombinant protein, emulsified in polyacrylamide gel as an adjuvant and boosted 21 days later with half the amount of antigen. It was maintained in the CDRI animal house facility and the experimental protocol was approved by the Institutional Animal Ethics Committee. Serum was collected from the blood of the immunized rabbit and stored at −20°C after adding 0.02% NaN_3.[29]

Detection of InhA using immunoprecipitation

Total cell proteins of mycobacteria were separated by 12.5% SDS–PAGE. The resolved proteins were stained with Coomassie Blue and replica gel was transferred to a nitrocellulose membrane for immunoblotting by a standard protocol.[29] The blot was probed with the anti-InhA antibody (1:1000 dilution) and developed with horseradish peroxidase-conjugated secondary antibody and diaminobenzidine as a substrate.[30]

Generation of spontaneous M. aurum mutants

In order to explore the isoniazid resistance mechanism in *M. aurum*, the isoniazid-resistant mutants of *M. aurum* were isolated from 32× the MIC of isoniazid (the MIC of isoniazid for *M. aurum* was found to be 1 mg/L). The culture containing 1.3×10^9 cfu of *M. aurum* was plated on nutrient agar containing 32 mg/L of isoniazid. Several small discrete colonies grew at a frequency of 1×10^{-7}. Ten colonies were purified and subcultured three times in isoniazid-free broth using limiting dilution of the culture.

Generation of genetically modified M. aurum mutants

Sense–antisense DNA technology was used to modify *M. aurum* strains genetically. This technology is based on the principle of modulating protein expression through transcribed mRNA using targeted sense and antisense DNA molecules.[31] Using this strategy sense–antisense InhA constructs were prepared by cloning the mt*inhA* gene in the pMV261 *E. coli*–mycobacteria shuttle vector, under the control of the hsp_{60} promoter at the BamHI site. Cloning of the mt*inhA* gene in the sense and antisense orientations in pMV261 was determined by restriction cleavage with SfiI which has a unique cleavage site present at base 220 in the mt*inhA* gene. In the sense orientation, a 600 bp fragment was generated with the vector fragment upon SfiI digestion while in the antisense orientation a 226 bp fragment was generated. The recombinant plasmids represent the sense and antisense InhA constructs [pMV261-1484s] and [pMV261-1484as], respectively. In order to generate genetically modified *M. aurum* mutants, these constructs were electroporated in *M. aurum* using 0.2 M glycine.[32] The immunoprecipitation assay was used to analyse the expression level of InhA protein in these mutants.

Testing of isoniazid and ethionamide susceptibility using the resazurin colorimetric assay in vitro

The modulation of isoniazid susceptibility in *M. aurum* mutants was examined by observing the viability of the prepared constructs with increasing concentrations of isoniazid using the redox indicator dye resazurin. A 0.2 mL aliquot of culture (0.3 OD_{600}) was dispensed in each well of a microtitre plate containing a 2-fold serial dilution of isoniazid. The plate was incubated at 37°C. After 24 h, 0.03 mL of resazurin (0.1% stock solution) was added to each well and the plate was reincubated for 3 h.[33] The MIC was defined as the lowest concentration of drug that prevented the change in colour from blue to pink indicating the viability of bacteria.

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Testing of isoniazid and ethionamide susceptibility within infected macrophages

The effective susceptibility to isoniazid and ethionamide was evaluated in the mouse monocyte macrophage cell line J744A.1 to ensure that an alteration of the intracellular drug susceptibility occurred in genetically modified *M. aurum* mutants. The J744A.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) at 37°C in 5% CO_2. The cells (10^6/mL) were mixed with *M. aurum*, *M. aurum* [pMV261-1484s] and *M. aurum* [pMV261-1484as] cultures at a ratio of 10 bacilli per cell. The suspension was allowed to form a monolayer in a 96-well plate. After 5 h of phagocytosis at 37°C, the monolayers were washed thoroughly with Hank's balanced salt solution containing gentamicin, to remove extracellular mycobacteria. They were then cultivated with DMEM containing FCS, in the presence and absence of drugs, at 37°C in 5% CO_2. After 72 h, infected macrophages were lysed with chilled 0.25% SDS and the cfu were determined by plating on NAT plates.[18,34]

Determining InhA interactions (in vitro) using circular dichroism (CD)

Intrinsic CD has been considered to be a valuable spectroscopic technique for studying protein–protein interactions in enzyme solution.[35] Based on this strategy, purified proteins and their complexes were used to measure the CD spectrum in the far UV (190–250 nm) at 20°C, with a Jasco J-810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The concentration used for proteins was 0.025 mM. Each spectrum is the average of three scans. The value obtained was normalized by subtracting the baseline recorded for the buffer, having a similar salt concentration as in the protein. The results were expressed as the mean residual ellipticity [θ], which is defined as $[\theta]=100\times\theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in moles of residue per litre, and l is the length of the light path in centimetres.[36]

Determining InhA interactions (in vivo) using BTH assay

In the BTH system the functional complementation between T25 and T18 fragments leads to the synthesis of cyclic AMP (cAMP) and in turn to transcriptional activation of the catabolic *lac* operon.[37,38] We used this *E. coli*-based two-hybrid system, consisting of bait–prey hybrid clones and β-galactosidase enzyme as a reporter marker, for observing *in vivo* interactions. As described above, the resulting pKT25 and pUT18C clones were used to construct all the possible co-transformants, each carrying a different pair of plasmids. To probe putative interactions, pKT25-mt*inhA* was co-transformed with the corresponding clones expressing *M. aurum* FAS-II enzymes into *E. coli cya* strain DHM1 (a strain deficient in endogenous adenylate cyclase of *Bordetella pertussis*). Ampicillin- and kanamycin-resistant transformants were selected from colonies grown on LB agar. The functional complementation due to interaction of proteins in these clones was estimated qualitatively by observing the blue colour of the colony in the presence of X-gal, and quantitatively by measuring β-galactosidase activity in the presence of ONPG as a substrate.[38] The positive controls were the pairs pKT25-zip and pUT18C-zip, and pKT25-mt*inhA* and pUT18-mt*fabD*, whereas the negative control was the pair pKT25-mt*inhA* and pUT18C-mt*sucA*.

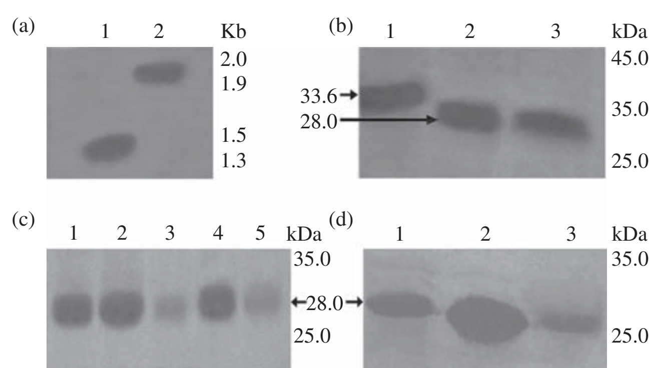

Figure 1. Detection of the InhA homologue. (a) Southern hybridization of SalI-restricted genomic DNA with the mt*inhA* gene: lane 1, *M. tuberculosis*; lane 2, *M. aurum*; λ DNA EcoRI/HindIII DNA marker. (b) Immunoprecipitation assay: lane 1, purified recombinant protein; lanes 2 and 3, total cell lysates of *M. tuberculosis* and *M. aurum*, respectively; protein MW marker. (c) Immunoprecipitation of the spontaneous *M. aurum* mutants (lanes 1–4) and *M. aurum* (lane 5) with anti-InhA antibody; protein MW marker. (d) Immunoprecipitation of genetically modified *M. aurum* mutants with anti-InhA antibody: lane 1, *M. aurum*; lane 2, *M. aurum* [pMV261-1484s]; lane 3, *M. aurum* [pMV261-1484as]; protein MW marker.

Results

InhA homology between M. aurum and M. tuberculosis

By Southern hybridization a single band was detected at ~1.9 and 1.3 kb in the DNA from *M. aurum* and *M. tuberculosis*, respectively (Figure 1a). This indicated the presence of a single copy of the *inhA* gene in the complex genome of *M. aurum*. Further, a single identical band of 28.0 kDa was detected by immunoprecipitation using anti-InhA antibody from total cell lysates of *M. aurum* and *M. tuberculosis* (Figure 1b). This suggests that the InhA homologue is present in *M. aurum*.

Upregulation of InhA in spontaneous M. aurum mutants

Using the broth dilution technique, all the isolated spontaneous *M. aurum* mutants were confirmed to have developed a stable resistance up to 100 mg/L of isoniazid. In the immunoprecipitation assay the differential intensity of a band of 28 kDa was detected in *M. aurum* and its isogenic isoniazid-resistant mutants using polyclonal anti-InhA antibody. This indicated that there was an upregulation of the InhA enzyme in isoniazid-resistant *M. aurum* mutants (Figure 1c, lanes 1, 2 and 4) as compared with *M. aurum* (Figure 1c, lane 5), suggesting the overproduction of the InhA enzyme in these spontaneous isoniazid-resistant mutants.

Upregulation of InhA in genetically modified M. aurum mutants

The immunoprecipitation expression analysis of the InhA enzyme in cell lysates of *M. aurum* [pMV261-1484s], *M. aurum* [pMV261-1484as] and parent *M. aurum* showed a distinct band of InhA protein in the control transformants and also a high level of InhA expression in the sense transformant, confirming the overproduction of InhA enzyme in the sense construct (Figure 1d).

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Modulation of isoniazid and ethionamide sensitivity in genetically modified M. aurum mutants, in vitro

In the resazurin drug susceptibility assay only sense *M. aurum* [pMV261-1484s] showed viable cells displaying pink colour up to 4 mg/L isoniazid, whereas the growth of parent *M. aurum* was inhibited at 1 mg/L isoniazid, showing blue colour. No change in the MIC of isoniazid was recorded for antisense *M. aurum* [pMV261-1484as] when compared with the parent *M. aurum* [Figure S1; available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. An increase in MIC was confirmed by survival of sense *M. aurum* [pMV261-1484s] in NAT supplemented with increasing concentrations of isoniazid, when compared with *M. aurum* and antisense *M. aurum* [pMV261-1484as] (Figure 2a).

Ethionamide susceptibility was also found to be modulated by the overexpression of mtInhA. We found that the parent and antisense strains of *M. aurum* survived at up to 20 mg/L, while the sense strain survived up to a concentration of 50 mg/L ethionamide (data not shown). The results showed a 4-fold increase in the MIC of isoniazid and a 2.5-fold increase in the MIC of ethionamide in the overexpressing strain as compared with wild-type *M. aurum*. Thus we can deduce that the overexpression of *M. tuberculosis* InhA protein in *M. aurum* increased the MIC of both isoniazid and ethionamide, whereas antisense transformants behaved like the parent strain.

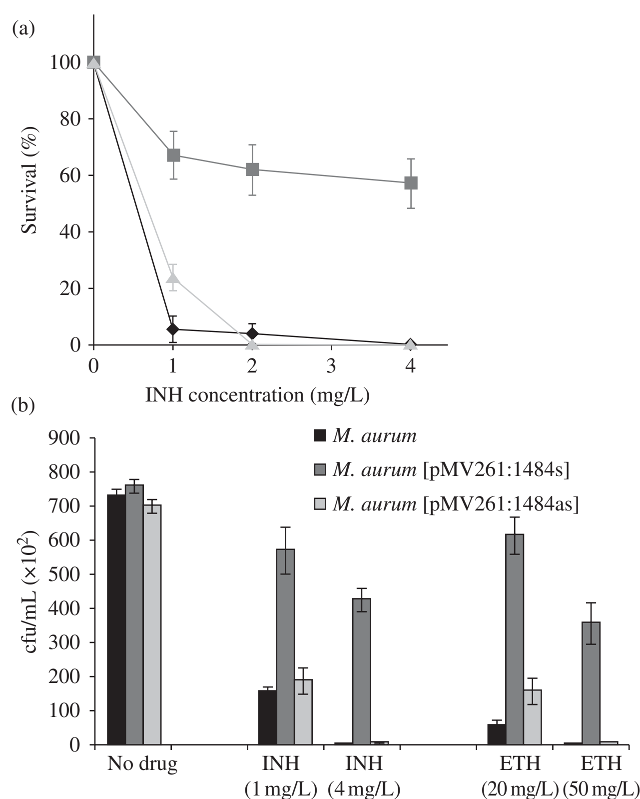

Figure 2. Drug susceptibility assays for genetically modified *M. aurum* mutants. (a) *In vitro* survival in the presence of isoniazid (INH). Diamonds represent *M. aurum*, squares represent *M. aurum* [pMV261-1484s] and triangles represent *M. aurum* [pMV261-1484as]. (b) *Ex vivo* survival in the presence of isoniazid as well as ethionamide (ETH). Each bar represents the mean value of cfu from three independent experiments. The standard deviation of the mean for each is shown as an error bar.

Modulation of isoniazid and ethionamide susceptibility in genetically modified M. aurum mutants, ex vivo

In the infected macrophage cell line J744A.1, the sense *M. aurum* survived up to 4 mg/L and 50 mg/L isoniazid and ethionamide, respectively, but the parent and antisense derivatives were found to be susceptible at 1 and 20 mg/L isoniazid and ethionamide, respectively (Figure 2b). There was 56% survival for sense *M. aurum* but only 1.27% survival for antisense *M. aurum* and 0.75% for *M. aurum* in the presence of 4 mg/L isoniazid. Similarly, we found 47% survival of sense *M. aurum* in comparison with 1.39% of antisense and 0.77% of parent *M. aurum* in the presence of 50 mg/L ethionamide (percentage survival was calculated from the cfu values given in Figure 2b). These data confirmed that the sense derivative of *M. aurum* was more resistant to isoniazid and ethionamide than the parent strain and antisense derivative in the intracellular environment as well.

Interactions of InhA with the kas operon of M. aurum, in vitro

On expression and purification of FAS-II enzymes, we obtained purified mtInhA, maFabD, maAcpM, maKasA and maKasB recombinant proteins of the expected size [Figure S2; available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. The CD spectra recorded for these purified proteins revealed them to be properly folded and rich in α-helices. We also observed differences in the mean residual ellipticity of the protein complexes and the average mean residual ellipticity of individual proteins at 208 and 222 nm that suggested structural changes in proteins when interacting (Figure 3, Table 2).

A very small difference was found for the complex of mtInhA and maFabD from the average curve obtained for the individual proteins (Figure 3a, Table 2). However, the highest percentage increase in ellipticity (64.07% and 60.04%) was observed for the mtInhA–maAcpM complex compared with the average of individual proteins (Figure 3b, Table 2). In the case of the mtInhA–maKasA complex, a decrease in helical content was observed (Figure 3c, Table 2), whereas an increase in helical content was obtained for the complex of mtInhA and maKasB (Figure 3d, Table 2).

Interactions of InhA with the kas operon of M. aurum, in vivo

Using the BTH system, the *in vivo* interaction of mtInhA with maFabD, maAcpM, maKasA, maKasB, maAccD6 and mtFabD was determined by the blue colour of colonies by comparison with positive and negative controls. A positive interaction between the two fusion proteins resulted in the production of the β-galactosidase enzyme, which cleaved the substrate X-gal to produce the blue colour phenotype. Blue colonies were observed in the case of maAcpM-, maKasA-, maKasB-, maAccD6- and mtFabD-interacting clones while pale blue colonies were noticed in case of the maFabD-interacting clone [Figure S3; available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Furthermore, the β-galactosidase assay confirmed high β-galactosidase activity for maAcpM-, maKasA-, maKasB-, maAccD6- and mtFabD-interacting clones, while a low activity was found for the maFabD-interacting clone compared with the positive control (Figure 4).

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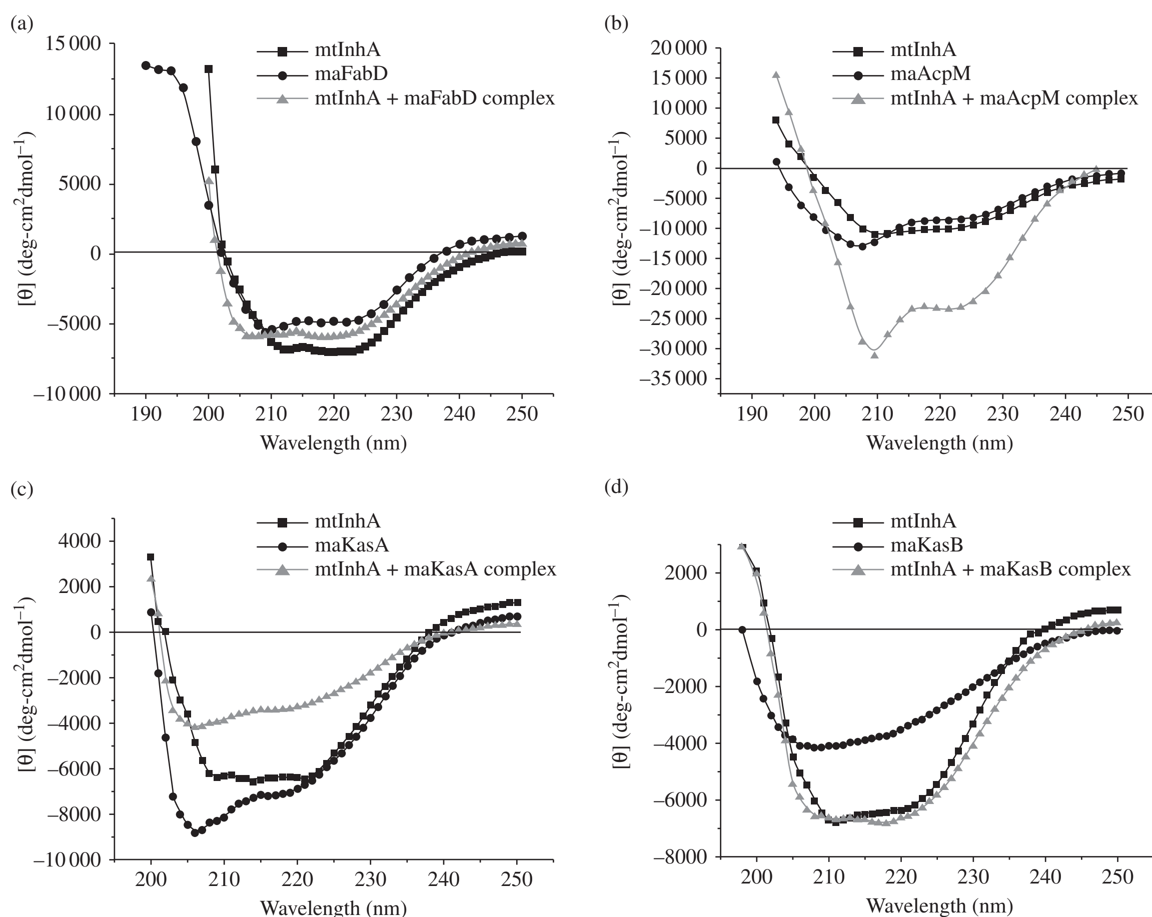

Figure 3. Circular dichroism spectrum showing structural changes in FAS-II-interacting complexes. (a) mtInhA–maFabD, (b) mtInhA–maAcpM, (c) mtInhA–maKasA and (d) mtInhA–maKasB in comparison with their individual protein components.

Table 2. Circular dichroism spectrum measurement for interacting recombinants showing mean residual ellipticity at 222 nm and 208 nm in degrees

Protein	θ_{222} MRE			θ_{208} MRE		
	average of individual proteins	complex	change of ellipticity (%)	average of individual proteins	complex	change of ellipticity (%)
mtInhA–maFabD	−5986.555	−5859.58	−2.10%	−5108.335	−5998.5	+14.83%
mtInhA–maAcpM	−9286.945	−23241.91	+60.04%	−11246.945	−31308.47	+64.07%
mtInhA–maKasA	−6446.135	−3138.91	−105.36%	−7329.15	−4038.85	−81.46%
mtInhA–maKasB	−4694.38	−6456.68	+27.29%	−5068.125	−6574.66	+22.91%

Discussion

Antimycobacterial chemotherapy includes two important drugs, isoniazid and ethionamide, both primarily targeting InhA, an important enzyme in the FAS pathway in *M. tuberculosis*.[8] In this study InhA mutants were developed in *M. aurum* to understand the mechanism of endogenous function of the InhA enzyme for these two drugs. The 2.5 h generation time, non-infectious nature and ability to survive intracellularly of *M. aurum* has already attracted attention, and thus motivated us to use it as a drug-resistant surrogate for primary screening of anti-TB inhibitors.[13,19]

Results from this study confirmed that an *M. tuberculosis* InhA homologue is present in *M. aurum* (Figure 1a and b). Earlier it was reported that the loss of acid fastness and mycolate contents occurs in isoniazid-treated *M. aurum*.[24,25,39] These findings indicated a possible involvement of InhA in the isoniazid resistance mechanism in *M. aurum*. With this knowledge, we developed spontaneous as well as genetically modified *M. aurum* mutants and investigated the endogenous expression level of the InhA enzyme. Our results indicated a multiple fold increase in isoniazid as well as ethionamide resistance, due to the overproduction of InhA enzyme in *M. aurum* mutants

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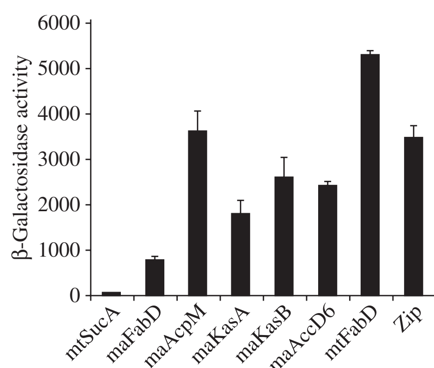

Figure 4. Bacterial two-hybrid analysis of FAS-II-interacting complexes. The mtSucA and Zip were taken as negative and positive controls, respectively. Each bar represents the mean value of β-galactosidase activity in Miller units from three independent cultures, each measured in duplicate. The standard deviation of the mean for each is shown as an error bar.

(Figure 2). Previous studies showed the same results for *M. tuberculosis*.[40] It is postulated, therefore, that the mechanism of isoniazid/ethionamide interference in InhA function is similar for *M. aurum* and *M. tuberculosis*.

Isoniazid interferes with the integrity of the mycobacterial cell wall by inhibiting the elongation of FAS, which is also accomplished by a number of other enzymes with different catalytic functions. These reside in two functional operons, the *inhA* operon (consisting of *inhA* and *mabA* genes) and the *kas* operon (consisting of *fabD*, *acpM*, *kasA*, *kasB* and *accD6* genes).[27] In *M. aurum* a similar organization of the *kas* operon and a high level of sequence similarities with *M. tuberculosis* orthologues at the DNA and protein level have also been reported.[21] Recently, Veyron-Churlet *et al.* have proposed the interaction of InhA enzyme with FabH, KasA, KasB, FabD and MabA enzymes of the FAS-II pathway in *M. tuberculosis*, stating that the InhA enzyme acts as a core molecule for communicating with other enzymes for the biosynthesis of fatty acids in *M. tuberculosis*.[10,41] With these considerations, we focused our investigation on understanding the role of the InhA enzyme in the FAS-II-interacting module and its functional networking with *kas* operon enzymes in *M. aurum*.

Interestingly, mtInhA interacts in a protein pair with all five enzymes of the *kas* operon which participate in FAS. We have demonstrated that there is a strong interaction of mtInhA with maAcpM, maKasB and maAccD6 (Figures 3 and 4). Of these, the interactions of AcpM and AccD6 enzymes with InhA are the first demonstration of a FAS-II-interacting module in mycobacteria. The higher β-galactosidase activity as well as the higher helical content of maAcpM–mtInhA, when compared with other enzymes, shows the closest association between maAcpM and mtInhA. However, we noticed that there is a lower association of mtInhA with maFabD in comparison with that of mtInhA with mtFabD (Figure 4). These observations indicate an equivalent InhA-mediated interaction network of the FAS-II pathway between *M. aurum* and *M. tuberculosis*, which further supports the use of spontaneous as well as genetically modified *M. aurum* mutants as surrogate strains of *M. tuberculosis* at the first step of *in vitro* and *ex vivo* drug screening. This study also gives new insights for using a fast growing surrogate in high-throughput exploration of novel chemotherapeutics causing a malfunction in two enzymes simultaneously. In addition, the BTH system, showing interactions of FAS-II enzymes, provides us with a future goal to establish a very convenient and specific screen in *E. coli* for isolating a new generation of active molecules targeting protein–protein interactions.

In conclusion, from our research results we infer that: (i) an *M. tuberculosis* InhA homologue is present in *M. aurum*; (ii) InhA is one of the molecular targets for isoniazid/ethionamide activity in *M. aurum*; (iii) InhA is acting as a core molecule and shows a close network of molecular interactions with *kas* operon enzymes of *M. aurum*; and (iv) the spontaneous and genetically modified *M. aurum* mutants are biologically relevant as a surrogate to isolate active inhibitors against drug-resistant mycobacteria. It is important that a genetically modified *M. aurum* surrogate will also contribute to the screening of FAS-II inhibitors specifically targeting the InhA enzyme. Using these inferences we have characterized the function of InhA in *M. aurum* and compared that with *M. tuberculosis*, in order to develop a model for high-throughput screening of inhibitors using a rapid method of MIC determination.[42]

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Transparency declarations

None to declare.

Supplementary data

Figures S1, S2 and S3 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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