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Protein–protein interactions within the Fatty Acid Synthase-II system of *Mycobacterium tuberculosis* are essential for mycobacterial viability

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Summary

Despite the existence of efficient chemotherapy, tuberculosis remains a leading cause of mortality worldwide. New drugs are urgently needed to reduce the potential impact of the emergence of multidrug-resistant strains of the causative agent *Mycobacterium tuberculosis* (*Mtb*). The front-line antibiotic isoniazid (INH), and several other drugs, target the biosynthesis of mycolic acids and especially the Fatty Acid Synthase-II (FAS-II) elongation system. This biosynthetic pathway is essential and specific for mycobacteria and still represents a valuable system for the search of new anti-tuberculous agents. Several data, in the literature, suggest the existence of protein–protein interactions within the FAS-II system. These interactions themselves might serve as targets for a new generation of drugs directed against *Mtb*. By using an extensive *in vivo* yeast two-hybrid approach and *in vitro* co-immunoprecipitation, we have demonstrated the existence of both homotypic and heterotypic interactions between the known components of FAS-II. The condensing enzymes KasA, KasB and mtFabH interact with each other and with the reductases MabA and InhA. Furthermore, we have designed and constructed point mutations of the FAS-II reductase MabA, able to disrupt its homotypic interactions and perturb the interaction pattern of this protein within FAS-II. Finally, we showed by a transdominant genetic approach that these mutants are dominant negative in both non-pathogenic and pathogenic mycobacteria. These data allowed us to draw a dynamic model of the organization of FAS-II. They also represent an important step towards the design of a new generation of anti-tuberculous agents, as being inhibitors of essential protein–protein interactions.

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Introduction

Tuberculosis is a major infectious disease that kills 3 million people each year. According to the World Health Organization, about 40 million people will die over the next 25 years (Anonymous, 2002). Among the multiple causes for these alarming statistics, there is the growing percentage (≈3%) of multidrug-resistant clinical isolates of the causative agent *Mycobacterium tuberculosis* (*Mtb*) in the world. In certain countries the prevalence of multidrug-resistant strains goes up to 40% (Anonymous, 2000). New drugs against *Mtb* are urgently needed. The most effective anti-mycobacterial drug in the last 50 years has been isoniazid (INH). INH targets the biosynthesis of mycolic acids (Banerjee *et al*., 1994; Dessen *et al*., 1995; Mdluli *et al*., 1998), which represent the major and most specific lipid components of the mycobacterial cell wall (Daffe and Draper, 1998).

Mycolic acids are very long chain (C_{60}–C_{90}) α-branched β-hydroxylated fatty acids whose biosynthesis is under the control of at least two discrete elongation systems, namely the eukaryotic-type Fatty Acid Synthase-I (FAS-I) and the prokaryotic-like FAS-II (Kremer *et al*., 2000; Asselineau *et al*., 2002). FAS-I consists of a multifunctional single polypeptide that performs *de novo* synthesis of medium length (C_{16}–C_{26}) acyl-coenzyme A (CoA). FAS-II catalyses the same type of reactions but it is composed of several distinct enzymes, it functions on acyl carrier protein (ACP) derivatives, and it is not capable of *de novo* synthesis in mycobacteria. The FAS-II initial substrates are medium length keto-acyl-ACP resulting from the condensation by the mtFabH protein of the acyl-CoA products of FAS-I with malonyl-ACP (Choi *et al*., 2000). After reduction by the keto-acyl-ACP reductase MabA (Banerjee *et al*., 1998), dehydratation by a yet unknown hydroxyl-acyl-ACP dehydratase, and reduction by the enoyl-ACP reductase InhA (Banerjee *et al*., 1994), either KasA or KasB catalyses the condensation with malonyl-ACP units in the FAS-II cycle (Schaeffer *et al*., 2001; Kremer *et al*., 2002; Slayden and Barry, 2002). Several pieces of data brought us to suspect

that the components of FAS-II might be tightly interconnected. First, a high-molecular-weight protein fraction displaying the FAS-II activity has been purified from the non-pathogenic bacterium *Mycobacterium smegmatis* (Odriozola *et al*., 1977). Second, both InhA and MabA have been shown to be present and active in this complex fraction (Marrakchi *et al*., 2000; 2002). Third, the complex pattern of action of INH on FAS-II that we recently showed to fully inhibit MabA *in vitro* with a mechanism comparable to that of InhA (Ducasse *et al*., 2004) was another clue to suspect the existence of an organized FAS-II macromolecular complex. InhA and KasA have been proposed to be the primary target for INH (Slayden *et al*., 2000) and even if the mechanism of inhibition by INH is described in more details for InhA, this question is still debated (Mdluli *et al*., 1996; Larsen *et al*., 2002; Kremer *et al*., 2003; Rawat *et al*., 2003). All these data may reflect a high degree of communication between the components of FAS-II.

An attracting hypothesis is that FAS-II would be organized in such a way that the sequential enzymes of this pathway are in close contact, allowing the elongating fatty acids to be passed directly from one catalytic site to another. The elongation of fatty acids might thus be a channelled process. The channelling of metabolic intermediates has received a strong support in the literature over the last 20 years (Srere, 1987; Ovadi *et al*., 2000). It has been demonstrated in the yeast mitochondrial Krebs tricarboxylic acid cycle and it is dependent on protein–protein interactions (Velot and Srere, 2000). Protein–protein interactions are essential in many cellular processes and as such, they have been used as targets for the search of potent inhibitors. For example, peptidic trans-dominant inhibitors were used to inhibit the pheromone response pathway of the budding yeast *Saccharomyces cerevisiae* (Caponigro *et al*., 1998; Geyer *et al*., 1999; Norman *et al*., 1999). These inhibitors were referred to as 'perturbagens' by analogy with 'mutagens'. A perturbagen, as defined by Caponigro *et al*. (1998), instead of inducing genes mutations, acts at the level of protein, disrupting specific biochemical interactions in cells to generate a mutant phenocopy.

The biosynthesis of mycolic acids is essential for mycobacterial viability (Vilcheze *et al*., 2000; Portevin *et al*., 2004) and the fact that protein–protein interactions could exist and might be involved in the channelling of this process has prompted us to search for such interactions among the known components of the FAS-II system. Thereby, we aim at defining a perturbagen that would pave the way for the discovery of a new family of anti-tuberculous drugs. By using a yeast two-hybrid (Y2H) strategy and co-immunoprecipitation (co-IP), we showed here that both homotypic and heterotypic interactions exist between the FAS II components of *Mtb*. We exploited the strongest interactions to design structural driven point mutations that were able to disrupt homotypic complexes and reveal heterotypic interactions. *In vivo*, these mutants are dominant negative in mycobacteria. This transdominant genetic analysis allows defining a perturbagen that targets the essential FAS-II complex of *Mtb*.

Results and discussion

Protein–protein interactions within FAS-II revealed by Y2H genetic analysis

Yeast two-hybrid approach is a powerful technique to identify protein–protein interactions. It has been shown that dissociation constants of ≈70 μM were sufficient to detect protein–protein interactions in GAL4-based two-hybrid assays (Yang *et al*., 1995). However, the detection of false positives still represents a major risk and the analysis has to be carefully controlled. To avoid this kind of artifacts, we took several basic precautions: first, we used a three-reporter gene system (HIS3, ADE2 and MEL1) in order to dispose of a range of stringency in the Y2H analysis. It is noteworthy that numerous true protein–protein interactions have been identified by using only one or two reporter genes (Lei *et al*., 1999; Steyn *et al*., 2002; 2003). The second precaution was to use multiple negative controls. Each gene was tested against the void corresponding vector and each gene was also assayed against the human Lamin protein which has been shown to be very poorly interactive and represent a valuable negative control (Ye and Worman, 1995). Each couple of protein was assayed in 'both' direction, i.e. as a fusion with binding domain (BD) and a fusion with activation domain (AD). And finally, after the selection of positive clones on selective plates by streaking yeast co-transformants, the results were confirmed by plating, counting and scoring of dilutions (1.10^4 and 1.10^3) of liquid cultures of each co-transformant as indicated in *Experimental procedures*.

The *kasA*, *kasB*, *inhA*, *mabA* and *mtfabH* genes from *Mtb* were cloned into the Y2H vectors pGAD-T7 and pGBK-T7. The resulting plasmids were used to construct all the possible AH109 co-transformants, each carrying a different couple of plasmids. As controls, AH109/pGBK-T7, AH109/pGAD-T7 and AH109/pGBK::*lam* were also transformed with the corresponding vectors expressing each FAS-II gene. The strains obtained were tested for their ability to grow on appropriate selective media to monitor the induction of the reporter genes HIS3 and/or ADE2. Individual co-transformants were streaked on DOBA-LT, DOBA-LTH and DOBA-LTA. An illustration of this principle is given in Fig. 1 where AH109/pGAD-T7::*kasA* was tested against pGBK-T7 derivatives.

The growth of each transformant was compared with the positive (AH109/pGAD::*AgT*/pGBK::*p53*) and the neg-

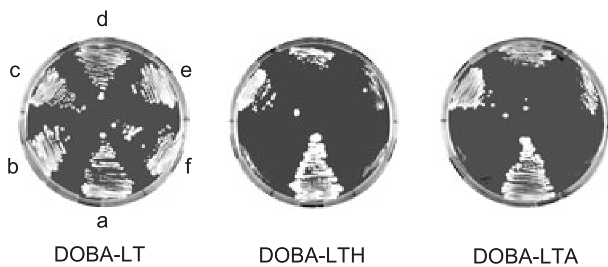

Fig. 1. Y2H first screen of protein–protein interactions between KasA and the *Mtb* FAS-II proteins. The derivatives of AH109 containing either pGAD::*AgT* and pGBK::*p53* (a), or pGAD::*kasA* and: pGBK::*lam* (b), or pGAD::*kasA* and pGBK::*kasA* (c), or pGAD::*kasA* and pGBK::*kasB* (d), or pGAD::*kasA* and pGBK::*inhA* (e), or pGAD::*kasA* and pGBK::*mabA* (f) were streaked on the selective plates DOBA-LT, DOBA-LTH and DOBA-LTA and incubated 2 weeks at 30°C.

ative (AH109/pGAD::*kasA*/pGBK::*lam*) controls. For KasA, this first screen was interpreted as a strong interaction with itself (quadrant c), a weaker interaction with KasB and InhA (quadrants d and e) and no interaction with either MabA or Lamin (quadrants f and b). After this first screen performed with each protein fused either to the AD or to the BD of GAL4, the final scoring of positives clones on four types of selective plates was performed by plating yeast cells from liquid cultures as indicated in *Experimental procedures*. In this final screen, the strongest interactions were identified by evaluating the maximum induction of the MEL1 reporter gene. A compilation of these data is presented in Table 1.

No growth was observed on any medium when each FAS-II protein was tested against either a void vector or the Lamin fusion protein indicating that these proteins did not activate any reporter gene and did not interact nonspecifically with Lamin. These negative data were omitted for clarity. The Y2H analysis revealed strong homotypic interactions for each protein. Four of them (KasA, InhA, MabA and mtFabH) were positive for the three reporter genes (HIS3, ADE2 and MEL1) indicating a strong specific interactions. The homotypic interaction KasB–KasB seemed to be the weakest because, in contrary to KasA–KasA, or InhA–InhA, or MabA–MabA, or mtFabH–mtFabH, the corresponding co-transformants of AH109 were MEL1$^-$. These results correlate with available biochemical and structural data. MabA, which displayed the strongest homotypic interactions, is able to form at least homodimers in solution and has been crystallized as a tetramer (Cohen-Gonsaud *et al.*, 2002). Similarly, InhA also displayed strong homotypic interactions and is known to form homotetramers (Dessen *et al.*, 1995). Finally, KasA and KasB are strongly related in sequence (67% identity) and also to the *Escherichia coli* orthologous condensing enzymes FabF (41% identity with KasA, 36% with KasB) and FabB (34% identity with KasB) (Schaeffer *et al.*, 2001) and, in this context, it is noteworthy that the FabF protein forms homodimers both in solution and during crystallization (Edwards *et al.*, 1997; Huang *et al.*, 1998). In the same manner, the homotypic interactions observed for mtFabH has to be correlated to the dimerization of the *Mtb* protein during crystal formation (Scarsdale *et al.*, 2001). This coherence between our results and available data on the quaternary structures of these enzymes assess the reliability of the Y2H method used here. More novels were the heterotypic interactions revealed by this approach (Table 1). First, The KasA–KasB interaction was a 'strong' interaction even revealed by the MEL1 reporter gene. Second, the interaction mtFabH–KasA was seen on 'both' directions with two reporter genes. Finally, mtFabH–KasB interaction was seen clearly only in one direction (when mtFabH was fused with the BD of GAL4), suggesting either that this interaction is weaker than the mtFabH–KasA interaction or that the structure of the mtFabH–KasB complex is very different from the mtFabH–KasA complex and imposes to the reconstituted GAL4 activator a strong structural constraint that prevents its activity when mtFabH is fused with the AD of GAL4.

In summary, we demonstrated that the three condensing enzymes of FAS-II are able to interact with each other. Another finding was given by the interaction pattern of InhA. It seemed that there is a connection between InhA and KasA which were both proposed to represent INH targets. This interaction was seen only with the ADE2 reporter gene and when InhA was fused with the BD suggesting either that KasA–InhA was a very weak interaction or that it imposed a very strong structural constraint to the reconstituted GAL4 activator. This interaction between InhA and the 'next' enzyme of the FAS-II cycle, if it is confirmed, is the first clue for the existence of channelling during the biosynthesis of long-chain fatty acids. This result has also to be brought together with a recent study of the mechanism of action of INH on InhA (Rawat *et al.*, 2003) where the possibility has been raised of a KasA–InhA interaction that might explain the occur-

Table 1. Y2H analysis of protein–protein interactions within FAS-II.

	BD fusions				
AD fusions	*kasA*	*kasB*	*inhA*	*mabA*	*mtfabH*
kasA	+ + ± [a]	+ + −	− + −	− − −	+ + +
kasB	+ + ±	+ + ±	− − −	− − −	+ ± ±
inhA	− − −	− − −	+ + +	− − −	± − −
mabA	− − −	− − −	− − −	+ + +	± − −
mtfabH	± ± ±	± − −	− − −	− − −	+ + +

a. Each of the three signs indicates the growth on DOBA-LTH, DOBA-LTA and DOBA-LTHA respectively. The significance of +, ± and – is given in *Experimental procedures*. Grey cells correspond to positive results on X-α-gal plates.

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rence of INH-resistant mutant in KasA, even if InhA is indeed the only primary target of INH. Surprisingly, we did not observe any interaction between MabA and the other components of FAS-II. Furthermore, InhA seemed to interact only with KasA. This was striking because we shown previously that both proteins were indeed present in a FAS-II crude fraction from *M. smegmatis* (Marrakchi *et al*., 2000; 2002). The first interpretation is that MabA do not interact with the other FAS-II components and that the InhA interaction with them is very labile. However, these two FAS-II proteins, in contrast to the others, have been shown to crystallize as tight tetramers (Dessen *et al*., 1995; Cohen-Gonsaud *et al*., 2002) and we thought that it could be a reason that could reduce heterotypic interaction in Y2H. To confirm the interactions revealed by the Y2H approach and test the latter hypothesis of a strong inhibitory effect of the homo-multimerization of MabA in Y2H, it was necessary to develop a biochemical approach.

The co-IP approach confirms and extends the interaction pattern of the FAS-II components

The vectors pGAD-T7 and pGBK-T7 are organized in such a way that the internal T7 promoter located just upstream from the multiple cloning site permits the *in vitro* transcription and translation of the gene of interest without including the AD or DB of GAL4. In addition, the translation products are tagged at their N-termini with either a haemagglutini (HA) or a c-Myc epitope giving, for example for KasA, proteins named h-KasA or c-KasA respectively. We evaluated the level of expression of each individual gene by loading on SDS-PAGE equivalent amounts (10 μl) of *in vitro* labelled transcription–translation reactions performed on equivalent amounts (1 μg) of plasmid expressing either h-proteins (Fig. 2A, column 1) or c-proteins (Fig. 2A, even columns). We did not observed any striking difference from one protein to another.

Labelled or non-labelled h-proteins were used to trap c-proteins on magnetic beads coated with anti-HA antibodies. When each labelled c-protein was incubated together with the coated beads without any h-protein, no significant background binding was observed (data not shown). All the combination of co-IP experiments were performed between KasA (row a), KasB (row b), InhA (row c), MabA (row d), mtFabH (row e) and the c-Lamin (Fig. 2A, column 2). We did not observe any background interactions between the five labelled h-proteins of FAS-II and the labelled c-Lamin protein (column 3) and concluded that the co-IP conditions were stringent enough to reveal specific interactions. When ‘cold’ h-proteins of FAS-II were used to trap labelled c-Lamin, no background binding of Lamin was observed on the beads

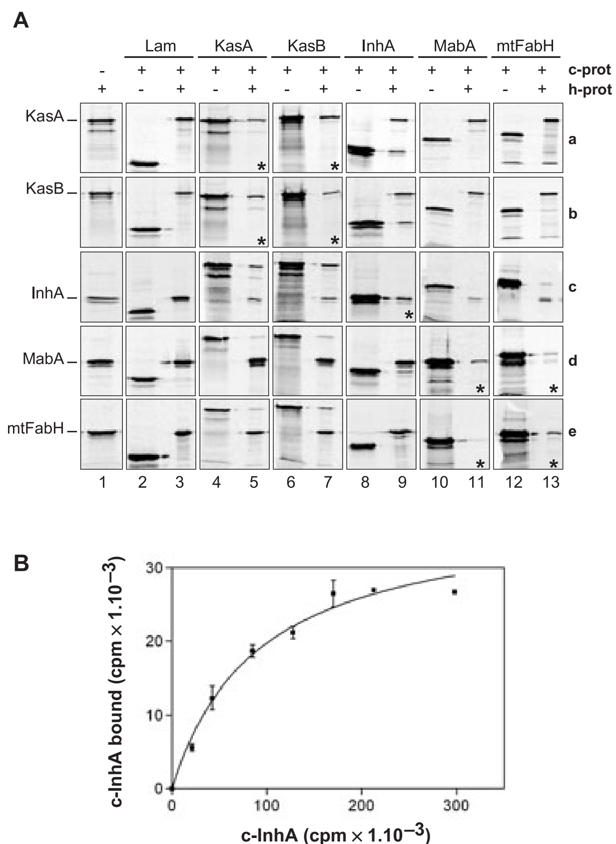

Fig. 2. Co-IP analysis of protein–protein interactions within the FAS-II system.
A. Analysis of co-IP between the *Mtb* FAS-II proteins. The L-[35S]-methionine-labelled h-proteins (HA tagged; h-prot) and c-proteins (c-Myc tagged; c-prot) from *in vitro* transcription/translation reactions and co-IP reactions products were fractionated by SDS-PAGE (10%) followed by phosphor-imaging analysis. In column 1, the gels contained the h-protein alone; h-KasA (row a), h-KasB (row b), h-InhA (row c), h-MabA (row d) and h-mtFabH (row e). The names of h-proteins are indicated in front of their position of migration. In even numbered column, the gels contained the c-protein alone. In the other columns (odd numbered except 1), the gel contained the co-IP reaction products performed between the c-proteins and the h-proteins. When the sizes of the proteins tested were two similar and in the case of the analysis of homotypic interactions, the co-IP reactions were performed between a labelled c-protein and a non-labelled h-protein; the corresponding panels are marked (*).
B. Co-IP analysis of KasA–InhA interaction. Increasing amounts of labelled c-InhA were incubated together with constant amount of labelled h-KasA (1.10^5 cpm) and the mix was subjected to standard co-IP experiment. After SDS-PAGE fractionation and quantification by phosphor-imaging, the quantities of InhA bound (in cpm) reported to 1.10^4 cpm of KasA bound was plotted against the amount of InhA (in cpm) present in the reaction. The curve was a fit using a one-site-binding curve fit after the law of mass action.

(data not shown). The latter conditions were used to search for the homotypic interactions. In each case a clear band corresponding to the labelled c-protein was trapped by the non-labelled corresponding h-protein (row a, column 5; row b, column 7; row c, column 9; row d, column 11; and row e, column 13). The homotypic inter-

actions previously detected with the Y2H analysis were confirmed for each protein of FAS-II.

All the interactions between the condensing enzymes were also detected. KasA and KasB (Fig. 2A, row a, column 7 and row b, column 5) were able to interact with each other whatever the 'direction' of the assay. However, the interactions observed between mtFabH (row e) and the Kas proteins (KasA, column 5, and KasB, column 7), even if it were largely above background, seemed weaker, especially when mtFabH was fused to the c-Myc epitope (column 13; rows a and b). Concerning the potential channelling of intermediates between the condensing enzymes and MabA, the co-IP results did not allow concluding. MabA (row d) remained very poorly interactive and a faint co-IP band was observed in only 'one direction' with KasA, KasB, InhA and mtFabH (columns 5, 7, 9 and 13 respectively). Together with the negative Y2H results for MabA, these results were not convincing enough to prove a real interaction between MabA and the condensing enzymes. In contrast, InhA (row c) seemed to really interact with these enzymes. A new interaction was revealed between KasB (column 7) and InhA (row c) and the KasA–InhA interaction seemed to be much stronger *in vitro* than it was in Y2H. These interactions were seen in 'both directions', i.e. between c-KasA and h-InhA (row c, column 5) and between h-KasA and c-InhA (row a, column 9). To ascertain that the KasA–InhA interaction was specific, we performed co-IP reactions between labelled h-KasA (1.10^5 cpm) and labelled c-InhA using increasing amount of c-InhA. The titration curve followed a one-site binding curve suggesting the existence of a specific binding of InhA onto KasA (Fig. 2B). This was an important finding which has to be correlated with the potential channelling of intermediates between InhA and KasA. Moreover, as it has been proposed (Rawat *et al.*, 2003), an interaction between these two proteins might represent a first explanation for the apparent contradiction in the literature that design both InhA and KasA as being the primary targets for INH (Mdluli *et al.*, 1996; Larsen *et al.*, 2002; Kremer *et al.*, 2003; Rawat *et al.*, 2003).

The results obtained with the co-IP assays were in agreement with the Y2H genetic data and particularly strengthen the fact that the three condensing enzymes are able to interact with each other and with the InhA protein. The KasA–KasB interactions observed here are supported by the study of the enzymatic activities of these two proteins (Slayden and Barry, 2002). *In vitro*, and in the presence of KasA, KasB displays a substrate preference for longer fatty acid chains than KasA suggesting a communication between these two enzymes and their successive involvement in the elongation of fatty acids by FAS-II. At this point, and because InhA interacts with the three condensing enzymes which interact with each other, we propose that three types of FAS-II complexes could exist, each formed by a distinct condensing enzyme and at least InhA. Either these complexes might coexist or the quaternary structure of a 'unique' FAS-II might change from one composition to another during the time and according to the degree of elongation of the substrate. The channelling of intermediates between these specialized FAS-II complexes might be ensured either by the interactions between the condensing enzymes or by the tight homo-multimerization of InhA itself. The apparent absence of MabA from this complex remains striking. In view of the structure conservation of the reductases InhA and MabA (Dessen *et al.*, 1995; Cohen-Gonsaud *et al.*, 2002), it was indeed surprising to observe such differences in the interaction pattern of the two proteins. However, this behaviour seemed to be highly correlated with the strength of the homotypic interactions displayed by the proteins and that would preclude heterotypic interactions.

Structure-based design and construction of point mutations of the mabA *gene*

The crystallographic co-ordinates of MabA (Cohen-Gonsaud *et al.*, 2002) were used in order to design subtle changes in the protein that would lead to the disruption of multimerization without, in theory, affecting its tertiary structure of the protein. The tertiary structure of MabA (247 residues) displays the same overall fold as the well-characterized InhA (269 residues) (Dessen *et al.*, 1995; Rozwarski *et al.*, 1999). This fold, which consists of a central parallel seven-stranded β-sheet flanked on both sides by α-helices, is characteristic of the short-chain dehydrogenase/reductase family of enzymes. MabA, like InhA, forms tetramers in solution and in the crystalline state (Dessen *et al.*, 1995; Cohen-Gonsaud *et al.*, 2002). The four subunits were labelled A, B, C and D respectively (Fig. 3A). The organization of the InhA and MabA homotetramers is also similar with a root mean square deviation of 2.6 Å for 646 matched Cα atoms. These tetrameric structures display two significant protein–protein interfaces that occur twice, because of internal symmetry leading to the existence of two types of dimers: A–B and C–D, or A–C and B–D (Fig. 3A). Furthermore, within each interface, the relatedness of the subunits by a twofold axis implies that the interactions between subunits are duplicated. The first type of interface in the MabA homotetramer is delineated by the C-terminal polypeptidic segment comprising helix α6 and strand β7 (Fig. 3B). This interface is mainly stabilized by hydrophobic and van der Waals interactions and involves few polar interactions. A salt bridge is formed between the carboxylate group of Asp228, located between α6 and β7, and the NH1 and NH2 atoms of Arg34 on α1 of the other subunit. The mutation of the aspartic acid in an arginine ($MabA^{D228R}$) might be detrimental to the stability of this interface as it

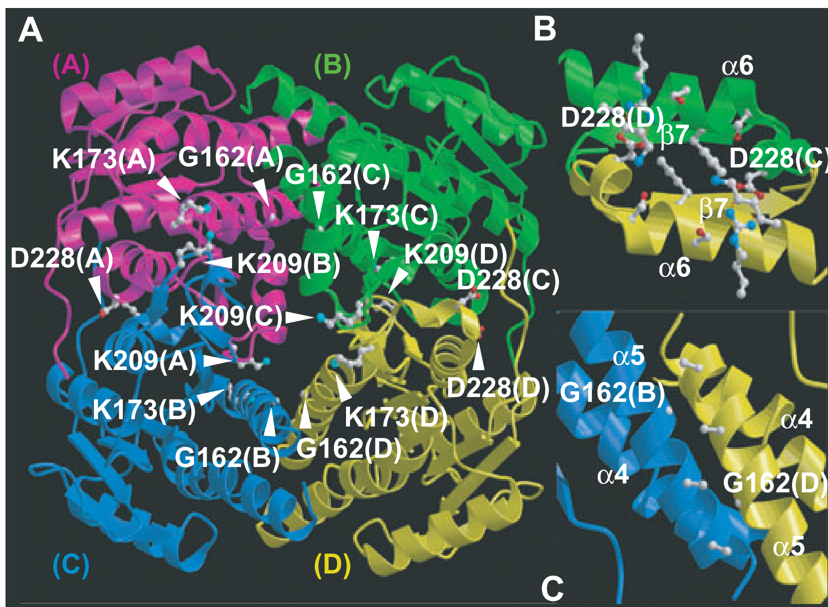

Fig. 3. Quaternary structure of MabA.
A. Ribbon representation of the MabA tetramer with the different subunits coloured in magenta (A), blue (B), green (C) and yellow (D). Side-chains of residues that were subjected to mutagenesis (G162 and D228) and of lysine residues that can be cross-linked (K173 and K203) are depicted. Oxygen, nitrogen and carbon atoms are in red, blue and grey respectively. Numbering is with one letter amino acid code followed by residue number and chain identifier between brackets.
B. α6–β7 interface between D and C subunits. Residues that were subjected to mutagenesis (D228) are represented.
C. α4–α5 interface between B and D subunits. Residues that were subjected to mutagenesis (G162) are represented.

would lead to severe steric and electrostatic incompatibilities with Arg34 and other surrounding residues (Lys10, Ser222, Phe223, Ser226). If this mutation is really detrimental, the mutant MabA^{D228R} is expected to form only A–B or C–D dimers. The second type of interface comprises helices α4 and α5, which together with helix α3 cover one side of the central β-sheet (Fig. 3C). This interface is also mainly hydrophobic in nature with few polar contacts. In MabA, the Cα atom of Gly162 on helix α5 is at van der Waals contact with the Cβ atom of Ala158 on helix α5 of the other subunit. This interaction would be impaired by any other amino acid than alanine at position 162 which would bump against Ala158 and also Ala154, and should have a profound destabilizing effect. A substitution for leucine was chosen (MabA^{G162L}). If this mutation is efficient, the mutant MabA^{G162L} is expected to form only A–C and B–D dimers.

Both single mutants and the double mutant (MabA^{G-D}), which is expected to remain monomeric, were constructed by site-directed mutagenesis directly on the plasmids pGAD::*mabA* and pGBK::*mabA*. As we previously shown with large amounts of pure proteins, only one type of MabA dimers (A–B or C–D) involving the interface α6–β7 can be cross-linked via two lysine residues (distance N$^{\zeta}$ K173–N$^{\zeta}$ K209, 5 Å) using glutaraldehyde (Cohen-Gonsaud *et al*., 2002). Several other lysine residue pairs could form intramonomer cross-links (e.g. K104-K105 and K131-K173; the latter competing with the interchain cross-reaction). With this approach, there is no possibility to cross-link the A–C or B–D dimers with glutaraldehyde or to reveal the tetramerization of MabA. When this type of reactions were performed on labelled *in vitro* transcription/translation product of wild-type *h-mabA*, we indeed observed a dimer migrating on SDS-PAGE at the previously observed (Cohen-Gonsaud *et al*., 2002) position of 60 kDa (Fig. 4A; D) showing that this technique can be used on proteins obtained *in vitro* for studying at least one type of dimerization of MabA (Fig. 4A). The mutation

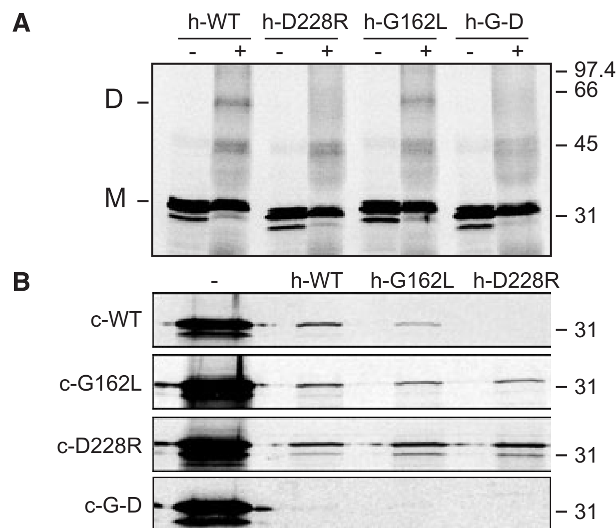

Fig. 4. Cross-linking and co-IP analysis of MabA mutants.
A. *In vitro* cross-linking of MabA variants. Phosphor-imaging analysis of SDS-PAGE (12%) fractionation of the *in vitro* transcription/translation-labelled products (10 μl) of pGAD-T7 derivatives of MabA (WT), MabA^{D228R} (D228R), MabA^{G162L} (G162L) and MabA^{G-D} (G-D) before (–) or after (+) glutaraldehyde cross-linking. Positions of monomer (M, 35 kDa), dimers (D, 60 kDa) are indicated. The position and size (kDa) of the molecular weight marker is indicated.
B. *In vitro* interactions between the MabA proteins. Co-IP analysis was as described in Fig. 2A. Labelled c-proteins derivatives of the four *mabA* alleles were loaded alone (left) or after co-IP with non-labelled h-MabA variants.

D228R, located in the α6–β7 interface of MabA and which was designed to disrupt the A–C and B–D dimers of MabA (Fig. 3B), completely abolished the formation of glutaraldehyde cross-linked dimers when it was present in either the single (MabA^{D228R}) or the double (MabA^{G-D}) mutant protein (Fig. 4A). It can be concluded that this mutation (D228R) was able to disrupt one type of dimerization and consequently tetramerization of MabA in solution. As discussed above, and because of the positions of the lysine residues in MabA, it is not possible, in theory, to cross-link the A–B or C–D dimers of MabA using glutaraldehyde (Fig. 3A). Because the G162L mutation was designed to disrupt these types of MabA dimers (A–B or C–D), there was, as expected, no visible inhibition of the glutaraldehyde cross-link of the A–C and B–D dimers (Fig. 3A) of the MabA^{G162L} protein (Fig. 4A). However, this result did not prove that this mutation (G162L) was inefficient.

When we screened each MabA single mutant using the Y2H system, we still observed interactions with wild-type MabA (Table 2). In Y2H, a dimer of MabA seemed sufficient to reveal an interaction. However, in contrast to wild-type MabA–MabA interactions, those involving the mutants of MabA were all negative on X-α-gal plates (data not shown). The MabA double mutant did not interact at all with the wild-type protein. This mutant behaved like a monomer indicating that the two mutations were sufficient to abolish the multimerization of MabA. Co-IP gave essentially the same results (Fig. 4B). The double mutant was completely unable to interact with either the wild-type or the single mutants MabA proteins. The overall strength of interactions observed for single mutants and wild-type proteins seemed to be affected. This was particularly clear when the mutant h-proteins were tested against the wild-type c-MabA. In this case, h-MabA^{G162L} was still able to interact with the wild-type c-MabA but this interaction seemed weaker than the interaction between the wild-type proteins. In addition, no interaction at all was observed between h-MabA^{D228R} and the wild-type c-MabA. Taking all data together, it might be considered that each mutations designed in the present study reduced the interactivity of MabA. Each mutation has been defined to perturb one type of dimer of the MabA tetramer. The effect of the D228R mutation which has been defined to disrupt the C–D and A–D interfaces of the MabA tetramer (Fig. 3) was clearly seen in Y2H, in cross-linking and in co-IP experiments. The effect of the G162L mutation which has been defined to disrupt the A–C and B–D interfaces of the MabA tetramer was seen in Y2H and co-IP and not in cross-linking because there is no possibility of glutaraldehyde cross-link between those subunits. Only the C–D and A–D subunits can be cross-linked via the lysine K173 and K203. The effect of both mutations in the double mutant was seen by the three methods. Here, we designed precise MabA mutations perturbing its overall quaternary structure and resulting in profound effects both *in vivo* and *in vitro*.

Table 2. Y2H analysis of MabA multimerization mutants.

AD fusions	BD fusions				
	kasA	*kasB*	*inhA*	*mabA*	*mtfabH*
mabA	− − −[a]	− − −	− − −	+ + +	± − −
mabA^{G162L}	+ + +	± − −	− − −	+ + +	+ + +
mabA^{D228R}	+ + +	± − −	− − −	+ + +	± − −
mabA^{G-D}	+ + +	+ − −	− − −	± − −	+ − −

a. Each of the three signs indicates the growth on DOBA-LTH, DOBA-LTA and DOBA-LTHA respectively. The significance of +, ± and – is given in *Experimental procedures*. Grey cells correspond to positive results on X-α-gal plates.

Mutations in MabA subunit interfaces reveal heterotypic interactions

Mutants of MabA were tested in the Y2H system against the other components of FAS-II and mtFabH (Table 2). Each mutation allowed to reveal interactions with KasA and with KasB, although slightly for the latter. This suggests that, at least in yeast, these interactions were probably masked by the high degree of homo-multimerization of MabA. In addition, only the G162L mutation allowed the detection of a significant interaction with MtFabH suggesting that the structure of the MabA–mtFabH complex is probably different from the ones formed with the other condensing enzymes (Table 2). These interaction patterns were clearly confirmed by co-IP. MabA as monomers (MabA^{G-D}) or as dimers (MabA^{G162L} and MabA^{D228R}) in solution can potentially interact with KasA, KasB, InhA and mtFabH (Fig. 5A). Quantification of the gels (Fig. 5B) obtained with the wild-type protein (Fig. 2A) and the different variants (Fig. 5A) indicated that any defect in the multimerization of MabA increased its affinity for each condensing enzyme and InhA. We propose that each monomer of MabA can interact with each condensing enzyme and that its high propensity to multimerization might maintain the macromolecular organization of several FAS-II complexes brought together.

MabA multimerization mutants as perturbagens of the FAS-II system

In addition to contributing to unravel the mode of action of the *Mtb* FAS-II complex, the interaction mutants of MabA provide a valuable tool to investigate, by transdominant genetics, the viability of mycobacteria after perturbation of the FAS-II complex organization. The *mabA* alleles were cloned into the vector pMV261 and the resulting plasmids were used to transform *M. smegmatis* mc^{2}155. The average transformation efficiencies at 37°C were sim-

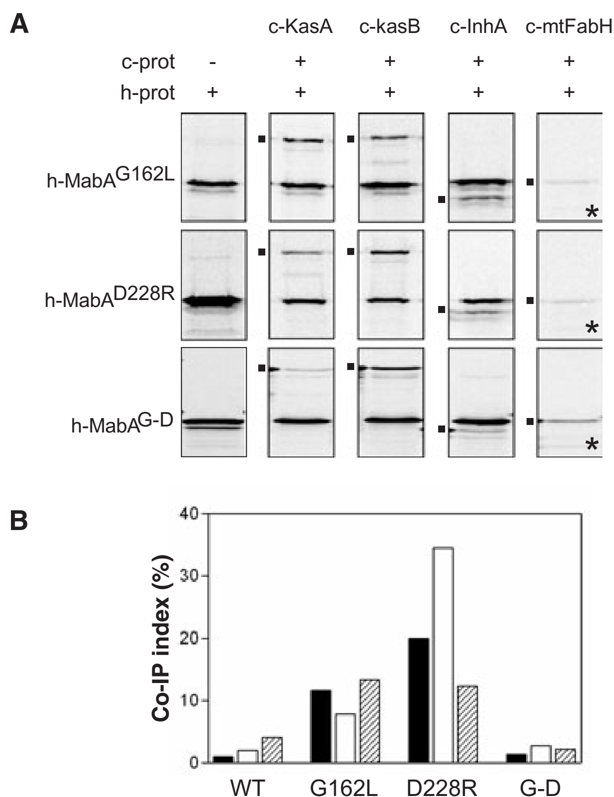

Fig. 5. *In vitro* interaction between MabA mutants and the FAS-II components.
A. Co-IP analysis of h-MabA variants with the FAS-II c-proteins. Results are presented as in Fig. 2A except that the analysis of c-proteins alone was omitted. The positions of migration of c-proteins (c-prot) and h-proteins (h-prot) are indicated with a black square and on the left side respectively.
B. Quantification of co-IP experiments between h-MabA derivatives and c-proteins of FAS-II. The co-IP index (in percentage) was defined as the ratio of the starting amounts of protein used in the reaction (h-protein/c-protein) reported to the final ratio of the proteins bound to the beads. The co-IP presented here was performed with h-MabA variants and c-KasA (black), c-KasB (white) or c-InhA (hatched).

ilar for pMV261, pMV261::*mabA*^{wt}, pMV261::*mabA*^{D228R} and pMV261::*mabA*$^{G\text{-}D}$ (Table 3). However, upon five independent trials, we did not observe any transformant with the vector expressing *mabA*^{G162L}. Because the pHSP60 promoter is slightly thermo-inducible (Stover *et al.*, 1991), we repeated these experiment at 30°C and 42°C. Except for the D228R mutation, the results were unchanged (Table 3). The *mabA*^{D228R} allele seemed to express its dominant negative effect only at 42°C. This transdominant genetic study was extended to the vaccine strain *M. bovis* BCG and the pathogenic strain *Mtb* H37Rv (Table 3). Because these two strains do not grow at 42°C, experiments were only carried out at 37°C. The mutant *mabA*^{G162L} was also dominant negative in both strains. The same experiments were carried out using an allele carrying the Y185L allele of MabA shown to be nearly inactive (28% of wild type) but capable of multimerization (Ducasse *et al.*, 2004). The *mabA*^{Y185L} allele was not dominant negative in any strain. The dominant negative effects observed with the G162L and D228R mutants is not likely to result from a non-productive diversion of either the substrate or the cofactor of MabA that would lead to a dead end in the biosynthesis of mycolic acids. This effect might rather be provoked by an interference of the protein brought *in trans* either with the resident wild-type MabA protein or with the FAS-II system itself via altered protein–protein interactions. The single mutant protein brought *in trans* might form unproductive dimers with the resident MabA wild-type protein and block the FAS-II system whereas the double mutant, which is not able to interact with MabA, has no effect. As this strong dominant negative effect was revealed with both single mutations whereas no effect was seen for the wild type or the double mutant, we postulate here that these mutants act as perturbagens of the FAS-II complex by modifying the degree of multimerization of MabA. The quaternary structure of MabA seems in turn to be determinant for the functioning of the FAS-II complex. These data point out the importance and the essentiality of protein–protein interactions within the FAS-II complex of *Mtb*.

In conclusion, in the present study, a complex network of interactions between the components of the essential FAS-II system of *Mtb* has been revealed. Our data converge to a working model of the functioning of the complex where several specialized FAS-II complexes would be interconnected by protein–protein interactions between the condensing enzymes and/or the core proteins comprising at least MabA and InhA and probably the not yet identified dehydratase (Fig. 6). If all the interactions revealed here were taken into account in a unique FAS-II complex, the resulting complex would be formed by at least a tetramer of MabA, a tetramer of InhA and three dimers of condensing enzymes (KasA, KasB and mtFabH). The molecular weight of this complex would not be in agreement with the value of 190 kDa proposed on early work on FAS-II (Odriozola *et al.*, 1977). Furthermore, it also would not be in agreement with the gel

Table 3. Transformation efficiencies of mycobacteria by pMV261::*mabA* derivatives.

	M. smegmatis			*M. bovis* BCG	*Mtb*
mabA alleles	30°C	37°C	42°C	37°C	37°C
None	≈1.10^{4}[a]	≈1.10^{4}	≈1.10^{4}	≈1.10^{5}	≈1.10^{5}
wild type	≈1.10^{4}	≈1.10^{4}	≈1.10^{4}	≈1.10^{5}	≈1.10^{5}
mabA^{Y185L}	≈1.10^{4}	≈1.10^{4}	≈1.10^{4}	Nd[b]	Nd
mabA^{G162L}	0	0	0	0	0
mabA^{D228R}	≈1.10^{4}	≈1.10^{4}	0	≈1.10^{5}	≈1.10^{5}
mabA$^{G\text{-}D}$	≈1.10^{4}	≈1.10^{4}	≈1.10^{4}	Nd	Nd

a. Expressed in cfu μg^{-1} of DNA.
Nd, not determined.

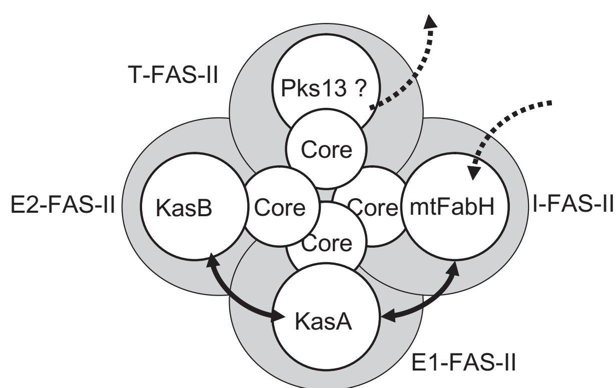

Fig. 6. Schematic representation of a model of interconnected specialized FAS-II complexes. Each putative specialized FAS-II complex is represented by a grey circle: I-FAS-II, initiation FASII; E1-FAS-II, elongation-1 FAS-II; E2-FAS-II, elongation-2 FAS-II; and T-FAS-II, terminal FAS-II. The core represents at least InhA, MabA and the non-identified dehydratase of FAS-II. Double-headed plain arrows represent observed interactions between the condensing enzymes. Stippled arrows symbolized the entry of acyl-ACP and the exit of mycolic-acids. Pks13 is the polyketide synthase 13.

filtration profiles of mycobacterial extracts used to purify active FAS-II fractions (Marrakchi *et al.*, 2000; 2002). A more attractive model, based on the interaction network revealed here, would be the existence of several types of specialized FAS-II complexes. We propose that either these complexes might coexist or the quaternary structure of a 'unique' FAS-II might change from one composition to another during the time and according to the degree of elongation of the substrate. We showed that mtFabH can interact with InhA and MabA suggesting the existence of an 'initiation-FAS-II' containing mtFabH and a 'FAS-II core' formed by InhA, MabA (and probably the dehydratase) that might allow the channelling of acyl-CoA from FAS-I to FAS-II during their condensation with malonyl-ACP by mtFabH. A second FAS-II specialized complex could be the 'elongation-1 FAS-II' (E1-FAS-II) formed by the 'core' and KasA because we showed that KasA can interact with both InhA and MabA. The acyl-ACP from the 'initiation-FAS-II' (I-FAS-II) would be transferred to this complex maintained in close contact via either the mtFabH–KasA interactions or the multimerization of the core proteins. In the same manner, the intermediate length acyl-ACP would then 'go' to the 'elongation-2 FAS-II' (E2-FAS-II) comprising the core and KasB that could end the synthesis of the meromycolic chain. The channelling of intermediates between these specialized FAS-II complexes might be insured either by the interactions between the condensing enzymes revealed here or by the tight homo-multimerization of MabA and/or InhA. In addition, we speculated the existence of a fourth type but hypothetic FAS-II specialized complex (Termination complex; T-FAS-II) formed by the same core and the recently identified terminal condensing enzyme Pks13 (Portevin *et al.*, 2004). This last complex might condense the acyl-ACP coming out of E2-FAS-II and acyl-ACP (or acyl-CoA) coming out of I-FAS-II. Experiments will be carried out in order to confirm this model and extend it to the other participants of the biosynthesis of mycolic acids like Pks13.

Another perspective of this work is the opening of a new field of investigation for the search of drugs directed against *Mtb*. Several human diseases (including cancer) occur as a consequence of the dissociation of particular protein–protein interactions (Sardet *et al.*, 1997; Dyson, 1998) and peptides that bind and affect a particular target have been identified (Colas *et al.*, 1996). The technology of selection of inhibitory peptide aptamers directed against cellular processes has been developed and optimized (Colas *et al.*, 1996; Blum *et al.*, 2000). Here, we have identified precise, specific and essential interactions whose disruption provokes the death of mycobacteria. This represents the first step towards the identification of a new generation of molecules that will be able to act as specific drugs directed against protein–protein interactions in the pathogen *Mtb*.

Experimental procedures

Strains and culture conditions

Plasmid constructions were performed in the *E. coli* K12 derivative Top10-F′ (Invitrogen). The Y2H recipient strain was *S. cerevisiae* AH109 from Clontech (*MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*HIS3*, *GAL2*$_{UAS}$-*GAL2*$_{TATA}$-*ADE2*, *URA3::MEL1*$_{UAS}$-*MEL1*$_{TATA}$-*lacZ*). AH109 was cultured in YEP (BIO101) with 2% dextrose and 0.003% adenine. Selective plates were made with synthetic medium DOBA (BIO101) supplemented with the amino acids of the Complete Supplement Mixture (BIO101) lacking leucine and tryptophan (DOBA-LT). The Y2H genetic tests were performed on DOBA-LT also devoid of histidine (DOBA-LTH), or adenine (DOBA-LTA), or both (DOBA-LTHA). *M. smegmatis* mc^{2}155 was cultured in TS (Tryptic Soy; Difco) and plated on TS-Agar. *M. bovis* BCG Pasteur (ATCC 35 534) and *Mtb* H37Rv (ATCC 25 618) were grown in Middlebrook 7H9 (0.05% Tween 80) and plated on 7H11 (Difco) with 10% Middlebrook Enrichment Medium ADC or OADC (BBL) respectively. When needed media were supplemented with kanamycin (50 μg ml^{-1}) or ampicillin (100 μg ml^{-1}). Electrotransformation of mycobacteria was achieved with a Bio-Rad Gene-Pulser (2.5 kV, 25 μFD, 1000 Ω).

Construction of the Y2H vector derivatives

The vectors pGBK-T7 and pGAD-T7 from the Matchmaker® Two-hybrid system 3 (Clontech) allowed the downstream cloning of genes in phase with the coding sequence of the BD or of the AD of the yeast GAL4 transcription activator. In pGBK-T7, the BD coding sequence is followed by the sequence of an internal T7 promoter and the coding sequence of an 11-amino-acid epitope tag from the proto-oncogene c-Myc, just upstream of the multiple cloning sites.

In addition, pGBK-T7 carries a kanamycin resistance gene and the yeast auxotrophic marker TRP1. After cloning of a gene of interest, the resulting protein will be a C-terminal fusion with the BD and the c-Myc epitope tag. The vector pGAD-T7 possesses the same genetic organization but it contains the coding sequence of the AD of GAL4, the sequence of an HA tag, an ampicillin resistance gene and the yeast LEU2 coding sequence. The five genes of *Mtb* (*kasA*, *kasB*, *inhA*, *mabA* and *mtfabH*) were amplified by polymerase chain reaction (PCR) from *Mtb* H37Rv chromosomal DNA using the *Pfu* DNA polymerase (Promega) and specific pairs of primers (Table S1) allowing their in-phase cloning in the multiple cloning sites of both pGAD-T7 and pGBK-T7. A description of the resulting constructs together with the description of the control vectors pGAD::*lam*, pGAD::*AgT* and pGBK::*p53* is presented (Table S2).

Y2H genetics analysis

AH109 has three reporter genes (HIS3, ADE2 and MEL1), under the control of three different GAL4-dependent promoters. The promoter driving the expression of HIS3 possesses a strong GAL4 Upstream Activating Sequences (UAS), and thus allows the detection of weak interactions. The promoter of ADE2 has a weak GAL4 UAS and thus allows only the detection of strong interactions. The third gene, MEL1, is responsible for the synthesis of α-galactosidase and is controlled by the natural and weak, GAL4-dependent MEL1 promoter region. AH109 was transformed with each couple of pGAD-T7 and pGBK-T7 derivatives as described (Clontech). Co-transformants, containing two plasmids, were selected on DOBA-LT. As a first screen for protein–protein interactions and for each couple of plasmid tested, at least five individual co-transformants were streaked on DOBA-LT with replicate on DOBA-LTH, DOBA-LTA and DOBA-LTHA. After this first screen, the validity of a given interaction was evaluated by plating dilutions (1.10^4 and 1.10^3) of saturated liquid cultures of an individual co-transformant on the four types of selective plates. On each medium, the number of colony-forming unit (cfu) was reported to the number of cfu on DOBA-LT. After the streak test and the plating assay, the scoring of a given interaction was performed as follows: an interaction was scored as positive (+) when numerous individual colonies were visible on the streak assay and when more than 80% of the population had the appropriate phenotype on a given medium in the plating assay. It was scored as plus-minus (±) when only a few colonies were visible in the streak assay and when 50–80% of the population had the required phenotype in the plating assay. In all the other cases, the results were scored as negative (–). The induction of MEL1 was evaluated by the coloration of colonies on DOBA-LT containing 20 μg ml^{-1} 5-bromo-4-chloro-3-indolyl α-D-galactoside (X-α-gal; Clontech). This test is not very sensitive and we counted as $MEL1^+$ the only colonies that displayed the same dark blue colour as the positive control strain AH109/pGAD::*AgT*/pGBK::*p53*.

Co-immunoprecipitation

In vitro transcription/translation of the genes of interest was performed with supercoiled DNA (1 μg) from pGAD-T7 or pGBK-T7 derivatives as matrix with the TnT® Quick Coupled Transcription/Translation System (Promega). Reactions were performed in a final volume of 50 μl either in the presence of 0.4 μCi μl^{-1} L-[^{35}S]-methionine (1000 Ci $mmol^{-1}$; Amersham) or with cold methionine (40 μM) to produce unlabelled h-proteins. For co-IP experiments, we used Dynabeads® M-450 Goat anti-mouse IgG coated with monoclonal anti-HA antibodies (Sigma). The ratios of the proteins were adjusted to 1:1 by evaluating the specific activity of each protein (in cpm per μl) and by correcting their differences in the number of methionine residues. Proteins were incubated with the coated beads (2 h at 4°C) in 20 μl of 50 mM Tris (pH 7.4), 50 mM NaCl and 0.025% Tween 20. After extensive washing of the beads with 100 mM Tris (pH 7.4), 100 mM NaCl and 0.025% Tween 20, the reactions were boiled in the SDS-PAGE loading buffer and fractionated on SDS-PAGE followed by autoradiography and Phosphor-imaging (STORM-Applied Biosystems).

Structure-based design and construction of point mutations of the mabA *gene*

Structural analysis was based on the crystallographic coordinates of MabA (Cohen-Gonsaud *et al.*, 2002). Site-directed mutagenesis was made directly on pGAD::*mabA* and pGBK::*mabA* using inverse-PCR amplification with self-complementary primers (Table S1) carrying the desired mutation. After *in vitro* transcription and translation, glutaraldehyde cross-linking of labelled h-proteins (10 μl) was performed as described previously (Cohen-Gonsaud *et al.*, 2002) and analysed on SDS-PAGE and Phosphor-imaging. For *in vivo* analysis of *mabA* mutant activity in mycobacteria, the five genes, *mabA*, $mabA^{Y185L}$, $mabA^{G162L}$, $mabA^{D228R}$ and the double mutant $mabA^{G\text{-}D}$, were cloned into the mycobacteria-*E. coli* shuttle vector pMV261 (Stover *et al.*, 1991) under the control of the pHSP60 signals carried by the vector (Table S2).

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mmi/mmi4334/mmi4334sm.htm

Table S1. Oligonucleotide sequences of PCR primers and cloning sites used for the construction of pGAD-T7 and pGBK-T7 derivatives.

Table S2. Description of plasmids.

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