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Occurrence of a putative ancient-like isomerase involved in histidine and tryptophan biosynthesis

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We report the occurrence of an isomerase with a putative $(\beta\alpha)_8$-barrel structure involved in both histidine and tryptophan biosynthesis in *Streptomyces coelicolor* A3(2) and *Mycobacterium tuberculosis* HR37Rv. Deletion of a *hisA* homologue (SCO2050) putatively encoding *N*′-[(5′-phosphoribosyl)-formimino]-5 amino-imidazole-4-carboxamide ribonucleotide isomerase from the chromosome of *S. coelicolor* A3(2) generated a double auxotrophic mutant for histidine and tryptophan. The bifunctional gene SCO2050 and its orthologue Rv1603 from *M. tuberculosis* complemented both *hisA* and *trpF* mutants of *Escherichia coli*. Expression of the *E. coli trpF* gene in the *S. coelicolor* mutant only complemented the tryptophan auxotrophy, and the *hisA* gene only complemented the histidine auxotrophy. The discovery of this enzyme, which has a broad-substrate specificity, has implications for the evolution of metabolic pathways and may prove to be important for understanding the evolution of the $(\beta\alpha)_8$-barrels.

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INTRODUCTION

The evolution of enzymes and metabolic pathways are intrinsically related (Copley & Bork, 2000; Teichmann *et al.*, 2001). Many enzymes involved in central metabolic pathways have $(\beta\alpha)_8$-barrel scaffolds, to which are attached different catalytic and substrate-binding folds (Gerlt & Babbitt, 2001; Todd *et al.*, 2001). The enzymes *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) isomerase (HisA, EC 5.3.1.16), which is involved in histidine biosynthesis (Lang *et al.*, 2000), and *N*′-(5′-phosphoribosyl)-anthranilate (PRA) isomerase (TrpF, EC 5.3.1.24), which is involved in tryptophan biosynthesis (Priestle *et al.*, 1987), are members of this structural family. It is generally accepted that the $(\beta\alpha)_8$-barrel enzymes involved in central metabolic pathways arose by divergent evolution (Copley & Bork, 2000; Henn-Sax *et al.*, 2001; Gerlt & Babbitt, 2001; Nagano *et al.*, 2002). Functional evidence for a common ancestry includes the directed evolution of the activity of TrpF from the HisA protein using random mutagenesis and selection *in vivo* (Jürgens *et al.*, 2000). TrpF and HisA catalyse an Amadori rearrangement of their cognate aminoaldoses into the corresponding aminoketoses (Fig. 1; Henn-Sax *et al.*, 2002).

The evolution of enzymes by divergence has a bearing on how biosynthetic pathways may have evolved to their current architecture. Among the hypotheses put forward on how metabolic pathways evolved the 'patchwork' hypothesis (Jensen, 1976) offers the most generally accepted explanation. This hypothesis states that metabolic pathways arose by the recruitment of enzymes with similar activity, and by subsequent modification of their substrate-binding ability. The model implies the existence of ancestral enzymes with broad substrate specificity, catalysing related reactions in different pathways. The recruitment of enzyme function, as inferred from predictions of the evolutionary relatedness of enzymes from different metabolic pathways (Parsot, 1987; Copley & Bork, 2000; Teichmann *et al.*, 2001) and detection of promiscuous activities (O'Brien & Herschlag, 1999) is widely acknowledged.

In *Escherichia coli* and its relatives, the enzyme PRA isomerase (TrpF), and the next enzyme downstream in the tryptophan biosynthetic pathway, indole-3-glycerol-phosphate (IGP) synthase (TrpC; EC 4.1.1.48), are present on a single peptide chain encoded by *trpC* (Priestle *et al.*, 1987). During our investigations into the regulation of tryptophan biosynthesis in *Streptomyces coelicolor* A3(2) (Hu *et al.*, 1999), we failed to discover a *trpF* gene next to *trpC*. This paradox of a function without a gene was confirmed after completion of the sequencing of the complete genome of *S. coelicolor* (Bentley *et al.*, 2002). A similar situation was encountered in the genomes of *Mycobacterium tuberculosis* HR37Rv (Cole *et al.*, 1998) and *M. leprae* (Cole *et al.*, 2001). Here, we report the occurrence of an isomerase, with a putative $(\beta\alpha)_8$-barrel structure predicted from its sequence, with a dual function in both histidine and aromatic amino-acid biosynthesis in *S. coelicolor* and *M. tuberculosis*. This report expands on the suggested physiological link between tryptophan and histidine biosynthesis observed in other organisms (Nester & Montoya, 1976). The discovery of a putative $(\beta\alpha)_8$-barrel enzyme, with a predicted ancient-like broad substrate specificity, may be relevant for understanding the evolution of this important structural family.

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RESULTS AND DISCUSSION

Functional genomics of *trpF in silico*

At present, the only actinomycete *trpF* sequence available in the database is that of *Corynebacterium glutamicum* (Matsui *et al.*, 1986). Sequence analysis using this sequence as a probe, either

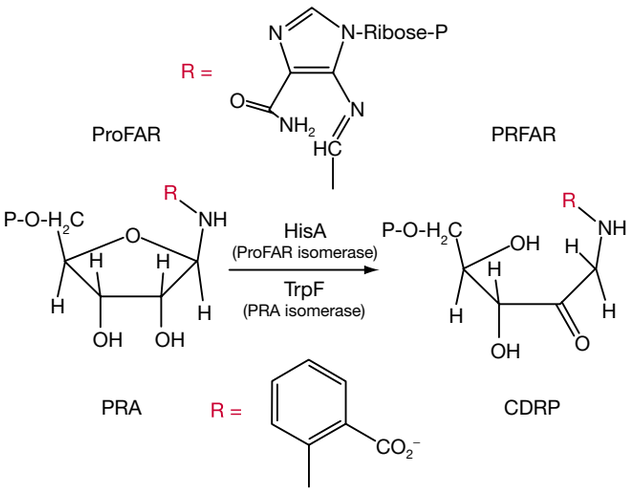

Fig. 1 | HisA and TrpF catalyse similar reactions. HisA and TrpF catalyse analogous Amadori rearrangements of *N*′-[(5′-phosphoribosyl)formimino]-5-aminoimidazole-4-carboxamide ribonucleotide (ProFAR) and *N*′-(5′-phosphoribosyl)anthranilate (PRA) into *N*′-[(5′-phosphoribulosyl) formimino]-5-aminoimidazole-4-carboxamide (PRFAR) and 1-[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate (CDRP), respectively. Red 'R's indicate the different side-chains in the two substrates.

using hidden Markov models (Karplus *et al*., 1998) or by carrying out BLAST searches (Altschul *et al*., 1997), failed to identify a homologue in *S. coelicolor* or the mycobacteria. It has been proposed in other organisms that TrpC and TrpF proteins share a common ancestry (Wilmanns *et al*., 1991; Gerlt & Babbitt, 2001). We tested whether the sequence of TrpC (open-reading frame (ORF) number SCO2039) of *S. coelicolor* could reveal a TrpF-like sequence from an actinomycete by performing PSI-BLAST searches (Altschul *et al*., 1997). Again, no TrpF-like sequence was identified. These observations indicate that the *trpF* genes of *S. coelicolor* and *Mycobacterium* species are of a different family to those found in other bacteria.

On the basis of the report by Jürgens *et al.* (2000) on the directed evolution of TrpF from HisA, we proposed that the HisA protein might have TrpF activity in *S. coelicolor*, which would account for the lack of *trpF* in this species. A *hisA* homologue was identified in *S. coelicolor* as part of a histidine biosynthetic cluster (Limauro *et al*., 1990). The completion of the genome sequencing project revealed that this cluster is localized on the chromosome upstream of a cluster of *trp* genes (Fig. 2A; Bentley *et al.,* 2002). A similar bifunctionality of *hisA* is implied in *M. tuberculosis* by the synteny of the *his* and *trp* clusters in this species (Fig. 2A).

Tryptophan and histidine *S. coelicolor* auxotrophy

We disrupted the *hisA* homologue of *S. coelicolor* M145 (ORF number SCO2050, according to the annotation of Bentley *et al.* (2002)); this was done by deleting two of the three catalytically important amino-acid residues, Asp 130 and Thr 166, which were identified by aligning the sequences of SCO2050 and *hisA* from *Thermotoga maritima* (Fig. 2B; Jürgens *et al.*, 2000; Henn-Sax *et al.,* 2002). This in-frame deletion was constructed in a way that avoided possible polar effects. As hypothesized, the resulting mutant (WH101) could not grow on minimal medium unless supplemented with both histidine and tryptophan (Table 1). We propose that SCO2050 should be renamed *priA* (*phosphoribosyl isomerase A*) to reflect the common effects of TrpF and HisA on phosphoribosylated substrates.

Gene complementation studies

The *priA* gene was inserted into the plasmid pIJ702 to make pIJ702–PriASc, which was used to transform WH101 mutants. The resulting transformants were prototrophic, showing that the tryptophan and histidine auxotrophy of WH101 mutants is exclusively due to the loss of functional *priA* (Table 1). We tested whether the *trpF* and *hisA* genes of *E. coli* could complement the WH101 tryptophan and histidine auxotrophies. The *E. coli* genes were cloned into the *Streptomyces* expression vector pIJ4123 under the control of the thiostrepton-inducible promoter. Thiostrepton-dependent expression of *trpF* (using pIJ4123–TrpFEc) in WH101 mutants restored tryptophan independence only, whereas expression of *hisA* (using pIJ4123–HisAEc) restored histidine independence only (Table 1). We also investigated the ability of *priA* to complement independent *hisA* and *trpF* deletions in *E. coli*. For this purpose, *priA* was cloned into the expression vector pGEX-4T-1 to form pGEX–PriASc, which was used to transform *E. coli* auxotrophs with mutations in *trpF* (W3110 *trpC* (*F*del); see Darimont *et al*., 1998), and *hisA* (Hfr G6; see Matney *et al*., 1964). Complementation of both mutations was achieved by expression of *priA* (Table 2). The *hisA* and *trpF* genes of *E. coli* cloned into pGEX-4T-1, to create pGEX–HisAEc and pGEX–TrpFEc, respectively, were used as controls (Table 2).

The intergenic complementation of *trpF* and *hisA* by *priA*, and the partial complementation of *priA* by either *trpF* or *hisA*, confirms that the product of *priA* is involved in the biosynthesis of both histidine and tryptophan in *S. coelicolor*. This discovery places into a physiological context the remarkable observation that TrpF and HisA activities can co-exist in a single protein (Jürgens *et al*., 2000) despite the low identity (10%) between the sequences of these enzymes. Preliminary characterization of the *in vitro* activity of PriA has shown that this HisA-like enzyme has PRA isomerase activity (data not shown).

Mycobacterium tuberculosis has a *priA* gene

To test the possibility that mycobacteria were similar to streptomycetes in having a single gene that encodes both TrpF and HisA activity, we cloned the putative *priA* orthologue (ORF number Rv1603, according to Cole *et al.* (1998)) of *M. tuberculosis* H37Rv into the expression vector pGEX-4T-1, to produce pGEX–PriAMt. This plasmid was shown to complement *hisA* and *trpF* mutations in *E. coli* (Table 2). As there is no *trpF* gene in *Mycobacterium* species, these results suggest that the protein product of Rv1603 in *M. tuberculosis* has the same metabolic role as that of PriA in *S. coelicolor*. This indicates that the presence of PriA in *S. coelicolor* and mycobacteria is due to the retention of ancient characteristics, rather than the modification of HisA to provide TrpF activity and a subsequent loss of TrpF. The failure to detect any *trpF*-like sequence in *S. coelicolor* supports the former interpretation.

Why did the streptomycetes and mycobacteria not develop independent *trpF* genes? In general, the streptomycetes do not regulate amino-acid biosynthesis by feedback repression of gene expression (Hodgson, 2000). Therefore, as enzyme expression is not co-ordinately regulated on a pathway-specific basis, enzymes with functions in multiple pathways are possible. The genome sequence of *C. glutamicum* shows that this actinobacterium has a *trp* operon

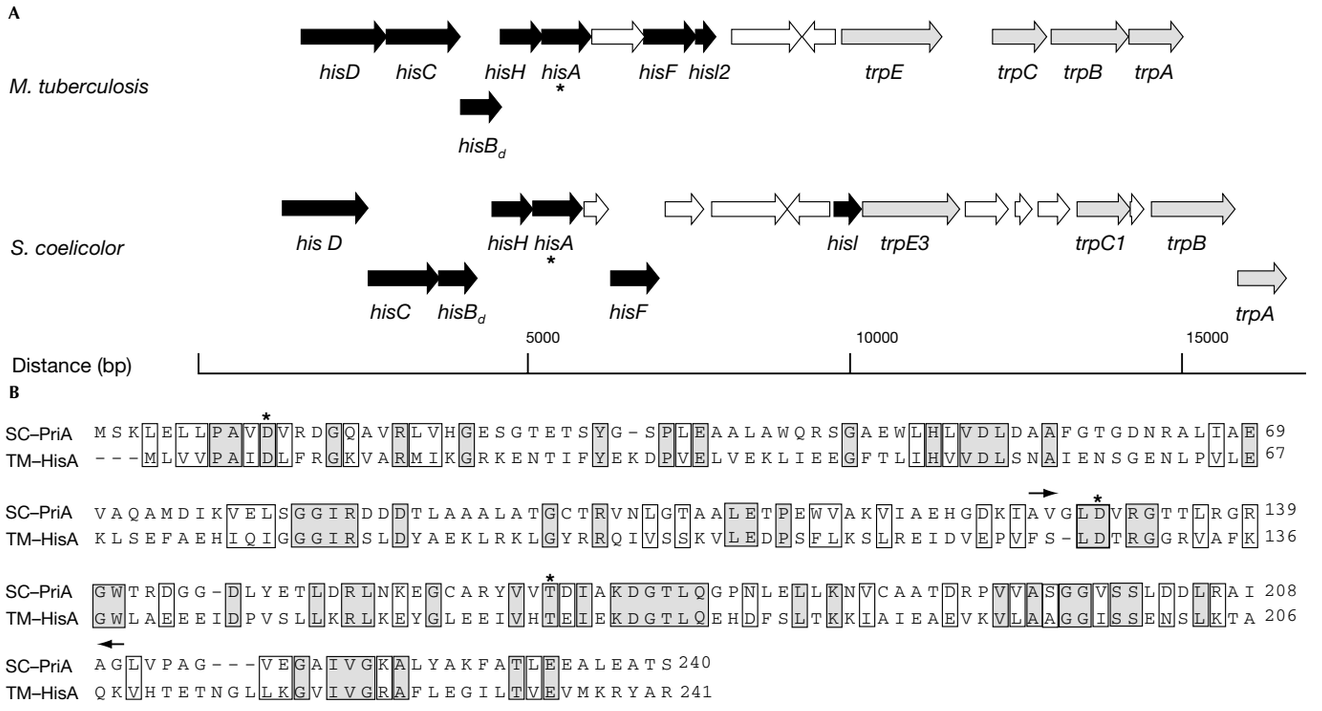

Fig. 2 | Synteny of the *his*/*trp* region and sequence similarity of PriA and HisA. (**A**) Organization of the *his*/*trp* cluster of *Streptomyces coelicolor* (Bentley *et al.*, 2002) and *Mycobacterium tuberculosis* (Cole *et al.*, 1998). The *hisA* homologues of *S. coelicolor* (SCO2050) and *M. tuberculosis* (Rv1603) are marked with asterisks. Genes involved in histidine (*his*; black) and tryptophan (*trp*; grey) biosynthesis are shown. (**B**) Sequence alignment of the HisA homologues of *S. coelicolor* (SC–PriA) and *Thermotoga maritima* (TM–HisA), which show 25% identity. Data from *T. maritima* (Jürgens *et al.*, 2000; Henn-Sax *et al.*, 2002) were used to identify the catalytically important residues in SCO2050 (Asp 11, Asp 130 and Thr 166 (asterisks)). The region of SCO2050 replaced in WH101 (this study) lies between Ala 126 and Gly 210 (indicated by arrows).

that contains a fused *trpCF* gene. This strain does regulate amino acid biosynthesis by feedback regulation (Sano & Matsui, 1987), and a simple explanation is that its ancestor acquired a complete, regulated *trp* operon after it separated from the streptomycete and mycobacteria evolutionary lines. Crawford (1989) has previously proposed this possibility. It would be interesting to test the putative *hisA* product of *C. glutamicum* for PRA isomerase activity.

An evolutionary interpretation of the nature of PriA

An obvious mechanistic explanation for the bifunctionality of PriA would be that it shows a broad specificity for the substrates PRA and ProFAR (Fig. 1). Enzymes with broad substrate specificity are not rare, and examples of these have accumulated since the postulation of the patchwork hypothesis by Jensen (1976). Nevertheless, the existence of broad substrate specificity, which stands at the core of the patchwork hypothesis, is usually inferred from the promiscuous activities of enzymes *in vitro* (for a review, see O'Brien & Herschlag, 1999) and/or overlapping specificities *in vivo*. Examples of the latter include members of the superfamily of aminotransferases (Jensen & Gu, 1996). This family includes another example of an enzyme that functions in both histidine and aromatic amino acid biosynthesis, which was first identified by Nester & Montoya (1976).

A number of enzymes involved in the biosynthesis of branched-chain amino acids show specificity for multiple substrates, such that isoleucine and valine are produced in the same metabolic pathway (Umbarger, 1996). However, this situation is different to that of PriA, because the substrates of PriA show marked difference in size and shape (Fig. 1). Therefore, if the broad substrate specificity of a given enzyme is in fact an ancient feature, as suggested by Jensen (1976), this implies that this feature has been retained in PriA throughout the course of its evolution. Thus, we conclude that PriA is a 'molecular fossil'.

Speculation

We believe that *priA* did not evolve as a consequence of loss of *trpF* and subsequent broadening of the specificity of the HisA protein. On the basis of the broad substrate specificity of PriA, one possibility is that *trpF* and *hisA* could have evolved from *priA* after gene duplication and specialization, as suggested by the patchwork hypothesis (Jensen, 1976). This would be an example of

Table 1 | Growth requirements of *Streptomyces coelicolor* WH101 and its transformants

Strain	Genotype	Growth requirements
WH101	*priA*::scar	Histidine and tryptophan
WH101(pIJ702)	*priA*::scar	Histidine and tryptophan
WH101(pIJ702–PriASc)	*priA*::scar (*priA*$^+$)	Prototrophic
WH101(pIJ4123–TrpFEc)	*priA*::scar (*trpF*$^+$)	Histidine
WH101(pIJ4123–HisAEc)	*priA*::scar (*hisA*$^+$)	Tryptophan

Table 2 | Complementation of *Escherichia coli* strains W3110 *trpC* (*F*del) and Hfr G6

Plasmid	Strain	
	W3110 *trpC* (*F*del)	Hfr G6
pGEX-4T-1	–	–
pGEX–PriASc	+	+
pGEX–PriAMt	+	+
pGEX–HisAEc	–	+
pGEX–TrpFEc	+	–

Complementation detected (+) or not detected (–) by expression of the insert from the *lacI*-dependent promoter induced with 10 μM isopropylthiogalactoside.

divergent evolution, and implies that the ancestor of *priA* encoded one of the older members of the $(\beta\alpha)_8$-barrel protein family (Fani *et al.,* 1994; Copley & Bork, 2000; Lang *et al.*, 2000), although it is also possible that the *priA* ancestor evolved relatively recently (Nagano *et al.,* 2002). An alternative possibility is that a TrpF function could have evolved from another enzyme, such as TrpC (Wilmanns *et al.,* 1991; Gerlt & Babbitt, 2001), allowing PriA to lose its TrpF activity, an example of convergent evolution. Therefore, convergent or divergent evolution may have accounted for the evolution of the extant $(\beta\alpha)_8$-barrel proteins.

METHODS

Computational sequence analysis and searches. The sequences used for the searches were TrpCF (accession number E24723), the genome of *C. glutamicum* (accession number NC_003450), TrpC (accession number SCO2039) and the genome of *S. coelicolor* (accession number NC_003888). The program ClustalW was used for sequence alignment.

Growth requirements of strains. Strains Hfr G6 and W3110 *trpC* (*F*del) were provided by the *E. coli* Genetic Stock Center. Minimal A medium (Miller, 1972) was used to test Hfr G6 and its derivatives, and modified Vogel–Bonner medium (Darimont *et al.*, 1998) was used to test W3110 *trpC* (*F*del) and its derivatives. Tryptophan and histidine were added to a final concentration of 100 μg ml^{-1}. Plasmids were selected for using ampicillin at 100 μg ml^{-1}; induction of *lacI*-dependent promoters was carried out using 10 μM isopropylthiogalactoside. The minimal medium for streptomycetes (Kieser *et al.*, 2000) was supplemented with tryptophan (37.5 μg ml^{-1}) and histidine (50 μg ml^{-1}) as appropriate. Thiostrepton (Sigma) was used at 50 μg ml^{-1} to select for plasmids and for induction of thiostrepton-inducible promoters. Apramycin (Sigma) selection was carried out using the antibiotic at 50 μg ml^{-1}.

Deletion of SCO2050 from the chromosome of *S. coelicolor*. The auxotrophic strain WH101 was constructed using REDIRECT® (Gust *et al.*, 2003). The protocol, plasmids and strains were provided by PBL Biomedical Laboratories. The oligonucleotides used for this were: 5′-TGGGTCGCCAAGGTCATCGCCGAGCACGGCGCAAGATCATTCCGGGGATCCGTCGACC-3′ and 5′-CTTCCCGACGATGGCCCCCTCGACACCGGCCGGACGAGTGTAGGCTGGAGCTGCTTC-3′ (the bases that are identical in the SCO2050 sequence are underlined). The disruption cassette was made by PCR using Expand high-fidelity DNA polymerase (Roche). SCO2050 was mutagenized in cosmid SC4G6 (Redenbach *et al.*, 1996) by homologous recombination (double crossover) replacing 255 bp from the 5′ end of the gene. The disruption cassette was removed by the FLP-recombinase system, leaving behind a 'scar' of 81 nucleotides with no stop codons. The newly mutagenized cosmid (carrying only the original marker) was re-engineered, inserting the selectable marker *aac(3)IV* and an RP4 *oriT*. The resulting construct was introduced into *S. coelicolor* M145 by RP4-based conjugation (Kieser *et al.*, 2000) and selected for using apramycin. Authentic double crossovers were obtained after two rounds of growth on fresh plates containing soya-flour mannitol medium (Kieser *et al.*, 2000) without selection. The replacement of SCO2050 was identified in colonies that were apramycin sensitive and auxotrophic for tryptophan and histidine. The presence of the wild type or of the mutated form of SCO2050 was detected by PCR using the primers 5′-GGGCGAAACCGAAGGACTC-3′ and 5′-TCGTGGCCGCCGTGGAGAACG-3′, and sequencing was used to confirm that the desired replacement event had taken place.

Cloning of the *hisA* and *trpF* genes of *E. coli*, and *priA* of *S. coelicolor* and *M. tuberculosis*. All DNA fragments were produced by PCR amplification, using primers with restriction sites engineered at their 5′ ends (see supplementary information online). PCR was performed using *Pwo* DNA polymerase (Roche). The PCR products were digested with the appropriate restriction enzymes and were ligated using T4 DNA ligase (Gibco) into the vectors pMTL22 (Chambers *et al.*, 1988), pET3a, pET22a (Novagen) or pGEX-4T-1 (Amersham Pharmacia). The resulting plasmids were used to transform *E. coli* MC1061 using the calcium method (Sambrook *et al.,* 1989). Fragments from restriction digests of the pMTL22 and pET constructs were ligated into plasmids pIJ702 and pIJ4123, and the resulting constructs were used to transform *Streptomyces lividans* TK24 protoplasts (Kieser *et al.*, 2000). The desired constructs were isolated from TK24 and used to transform *S. coelicolor* WH101 protoplasts. The *priA* gene (SCO2050) from *S. coelicolor* was amplified by PCR from cosmid 4G6. The *hisA* and *trpF* genes of *E. coli* were amplified from chromosomal DNA. The *trpF* gene was also cut out of pMS401 (a gift from M. Samaddar and J. Blackburn) using *Nco*I and *Bam*HI and sub-cloned into pET22a, from where *trpF* was subsequently removed using *Nde*I and *Bam*HI and subcloned into pIJ4123. The *priA* orthologue (Rv1603) of *M. tuberculosis* H37Rv was amplified from chromosomal DNA, provided by D. Roper. Sequencing was used to confirm all constructs.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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