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A split-protein sensor for studying protein–protein interaction in mycobacteria

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ABSTRACT

The study of protein function in living cells is an essential complement to genomics, yet method development does not always keep pace with sequencing. Experimental techniques for the genus mycobacteria are relatively underdeveloped, though seventeen genomes have been sequenced. "Split-Trp" is a split-protein sensor used to detect protein–protein interactions in tryptophan auxotrophic *Saccharomyces cerevisiae*, but the principles behind the sensor should allow it to function in a broad range of microbial hosts. Here we introduce Split-Trp to *Escherichia coli* and *Mycobacterium smegmatis* and demonstrate that this system is a simple assay for protein interaction in both organisms.

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1. Introduction

Mycobacterium tuberculosis, the causative agent of TB, is responsible for 1.7 million deaths per year. Control of TB by vaccination and by chemotherapy is increasingly undermined by the synergy of this pathogen with the Human Immunodeficiency Virus, and the increasing prevalence of multi-drug resistant strains (Dye, 2006). Considerable effort is being directed at understanding the biology of this intractable and slow growing bacterium, with the goal of producing an improved vaccine and new drug treatments. Since the publication of the complete genome of *M. tuberculosis* H37Rv in 1998 (Cole et al., 1998), a further sixteen genomes of pathogenic mycobacteria have been published, and the genomes of 15 other pathogenic and non-pathogenic strains are in progress (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Functions have been assigned to half of the ~4000 proteins predicted from the genome of *M. tuberculosis* H37Rv (Camus et al., 2002), leaving the function the remaining proteins to be determined experimentally. There has been great progress in the development of efficient vectors and methods for gene knockout by allelic exchange (Pelicic et al., 1997), but the identification of interactions between mycobacterial proteins, or between mycobacterial virulence proteins and host proteins, remains a difficult task, often relying on surrogate hosts such as the yeast two-hybrid system. New methods to study protein interactions within either mycobacteria themselves or appropriate bacterial hosts would facilitate investigation of the biology of these important pathogens.

Split-protein sensors, also called protein fragment complementation assay (PCA), have become an important tool for studying protein interactions in living cells (Piehler, 2005). These sensors have several advantages over the yeast two-hybrid system, including the ability to work with membrane proteins. The Split-Trp protein sensor was selected here for development in mycobacteria because of its simple growth assay and potential applicability to any host with a tryptophan biosynthetic pathway (Tafelmeyer et al., 2004). The sensor is derived from a 24 kDa monomeric enzyme that catalyses the isomerization of *N*-(5′-phosphoribosyl)-anthranilate in the biosynthesis of tryptophan in *Saccharomyces cerevisiae*. Fragmentation of this enzyme at residue 44 gave rise to peptides N_{trp} and C_{trp} that only reconstitute enzyme activity when fused to interacting proteins (Fig. 1). Protein-protein interactions can thus be detected as they enable the growth of Trp1p deficient *S. cerevisiae* strains on media lacking tryptophan (Tafelmeyer et al., 2004). Here we describe the introduction of the Split-Trp assay in *E. coli* and also in *Mycobacterium smegmatis*, a fast-growing, non-pathogenic model for *M. tuberculosis*. This system complements a recently described split-protein sensor based on mouse dihydrofolate reductase (mDHFR), adapted for use in *M. smegmatis* (Singh et al., 2006), where the read-out is growth of bacterial colonies in the presence of trimethoprim to selectively inhibit the bacterial DHFR.

2. Results and discussion

2.1. Detection of protein interactions using Split-Trp in E. coli

As a first bacterial host for the Split-Trp assay we used a tryptophan deficient strain of *E. coli*, Δ*trpF* (Sterner et al., 1995). A pair of compatible plasmids was constructed to allow co-expression of the Split-Trp fragments in *E. coli*. PL285 encodes N_{trp} followed by a flexible amino acid linker and a cloning site to insert the gene of interest

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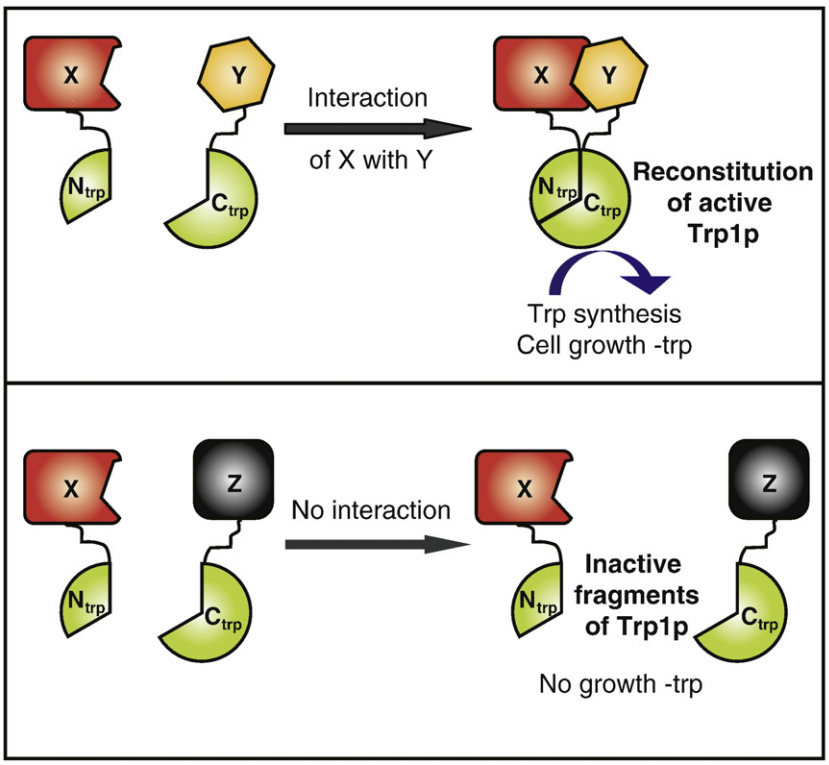

Fig. 1. The principle of the Split-Trp sensor for protein interactions. N_{trp} is fused to the N-terminus of protein X and C_{trp} is fused to the C-terminus of protein Y, resulting in proteins N_{trp}-X and Y-C_{trp}, with peptides of 8 amino acids linking the fused proteins. Interaction between proteins X and Y leads to the reconstitution of active Trp1p and the complementation of tryptophan auxotrophy. Co-expression of N_{trp}-X and Z-C_{trp} does not complement tryptophan auxotrophy, since protein X does not interact with Z.

(Fig. 2). PL284 encodes C_{trp}, and the gene of interest is cloned as an N-terminal fusion, again joined by a flexible amino acid linker. Expression of each fusion protein is from the lac promoter, and each protein carries the HA epitope tag for quantitation by Western blotting. To demonstrate that protein–protein interactions can complement tryptophan auxotrophy we chose the well-known rapamycin-mediated interaction of FK506 binding protein (FKBP) with the FKBP-rapamycin binding domain of FRAP (FRB). We also cloned peptides C1 and C2 that form a coiled-coil (Oakley and Kim, 1998; Tafelmeyer et al., 2004). *E. coli* Δ*trpF* were transformed with pairs of test plasmids and grown in media containing tryptophan, then washed in water and spotted onto minimal medium lacking tryptophan. In parallel cells were spotted onto the same medium with additional rapamycin or additional tryptophan. All strains grew on the medium containing tryptophan, indicating equal numbers of viable cells in each pre-culture. On the medium lacking tryptophan, only the strain expressing N_{trp}-C2 and C1-C_{trp} was able to grow. The association of C1 and C2 into a coiled-coil led to the assembly of the Split-Trp fragments into active Trp1p, which complemented the tryptophan deficiency (Fig. 3A). By contrast, the strain expressing N_{trp}-FRB and FKBP-C_{trp} only grew on medium lacking tryptophan when plates were supplemented with rapamycin to induce dimerization. As a control, *E. coli* Δ*trpF* expressing N_{trp}-C2 and FKBP-C_{trp} were spotted on the same plates, and these cells were unable to grow on media lacking tryptophan.

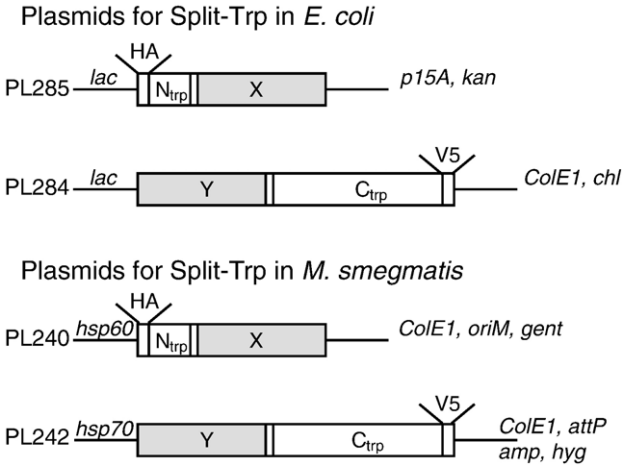

Fig. 2. Diagram of the Split-Trp plasmids used in this work. PL285 and PL284 are compatible episomal plasmids used for co-expression of N_{trp} and C_{trp} fusion proteins in *E. coli*. PL240 is an episomal mycobacterial-*E. coli* shuttle plasmid for expression of N_{trp} fusion proteins in *M. smegmatis*, and PL242 is an integrating mycobacterial-*E. coli* shuttle plasmid for expression of C_{trp} fusion proteins in *M. smegmatis*. The potential interaction partners (genes of interest X and Y) are shaded in grey. The length of each gene fragment is drawn to scale, with the genes of interest depicted as 300 base pairs. HA and V5 are epitope tags included to allow verification of protein expression by immunoblotting.

2.2. Protein interactions in two-component systems

To validate the assay with more diverse proteins including membrane proteins, we made fusions of the Split-Trp fragments with two component system proteins from *M. smegmatis*. Histidine kinase KdpD is an integral membrane protein that phosphorylates the cognate response regulator KdpE, as part of a potassium sensing system. An unrelated response regulator CpxR from *E. coli* was used as a control. The association of KdpD with its substrate KdpE functionally reconstitutes Split-Trp, allowing growth on minimal media, whereas KdpD does not associate with CpxR (Fig. 3B). Histidine kinases and response regulators are known to form homodimers, so it was expected that association of N_{trp}-KdpE with KdpE-C_{trp}, N_{trp}-KdpD with KdpD-C_{trp} and N_{trp}-CpxR with CpxR-C_{trp} would also lead to growth on minimal media, as seen in Fig. 3B. In addition to the

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