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Coupling reporter expression to respiration detects active as well as dormant mycobacteria *in vitro* and in mouse tissues

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ABSTRACT

Background: *Mycobacterium tuberculosis* is known to slow down its transcriptional activity during dormancy. Hence, while using reporter strains, it is important to couple the reporter gene to a promoter that is strong and sensitive both in active and dormant *M. tuberculosis*. Since respiration is an indispensable process even in dormant bacteria, validation of the promoters of respiratory chain genes – type II NADH dehydrogenase (P_{ndh}) and adenosine triphosphate (ATP) synthase operon (P_{atps}) – of MTB was undertaken for this purpose.
Methods: Putative promoter containing sequences were cloned upstream of a red fluorescent protein (RFP) gene. *Mycobacterium smegmatis* or *M. tuberculosis* carrying episomal constructs were validated for growth, fitness and fluorescence in different models *in vitro* and *in vivo*.
Results: Either promoter can drive stable and strong expression of RFP in actively growing and dormant *M. smegmatis in vitro* without significantly affecting growth or viability. Fluorescence due to P_{ndh} and P_{atps} was significantly higher than P_{hsp60}. The fitness of *M. tuberculosis* H37Rv counterparts was unaffected inside J774 macrophages. In immunocompetent mice, despite an initial attenuation in the lungs, both strains reached loads similar to wild type during chronic infection. In the spleen, the fluorescent strain counts were similar to wild type counts throughout. RFP fluorescence in tissue homogenates was more homogenous among mice due to P_{ndh} compared with P_{atps}.
Conclusions: Coupling an appropriate reporter to the promoter of *ndh-2* gene of *M. tuberculosis* can make the reporter expression respiration sensitive and thereby reliably detect both active and dormant populations of the reporter strain.

Introduction

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) continues to be a global emergency. According to the World Health Organisation (WHO) Report 2012, there were 8.7 million TB cases in the year 2011, two thirds of which were new cases. Twenty percent of previously treated cases and 3.7% of new cases are thought to have multidrug-resistant

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(MDR)-TB, 9.0% of which are potentially extensively drug resistant (XDR) [1]. In this backdrop, research on (MTB) continues to develop novel, cost-effective drugs and to shorten the duration of treatment. Screening of molecules for anti-tubercular activity starts with *in vitro* assays measuring the viability of the laboratory strain MTB H37Rv in the presence of the inhibitor. Lead molecules selected for pre-clinical testing are tested in a mouse model of TB, where the ability of the compound to reduce the bacillary loads in lungs and spleen is the desired parameter. In laboratory media, mycobacteria in general are slow growers, with generation times ranging from 3 to 24 h [2]. Colonies of laboratory strains of MTB appear between 12–14 days if sub-cultured from laboratory media and can take up to 3–4 weeks if the bacteria are being isolated from an infected host. The conventional colony forming unit (CFU) assay used for estimating MTB loads in mouse organs are therefore time consuming.

Faster and more sensitive detection of mycobacteria in cultures started in the 1990s with the help of recombinant strains of MTB expressing fluorescent and bioluminescent reporter proteins. Firefly luciferase was used for detection of MTB growth for the first time in the early 1990s by either expression from mycobacteriophage or from episomal plasmid [3,4]. Bioluminescent reporter MTB has been developed recently for optimal *in vivo* non-invasive imaging in mice [5].

Use of fluorescent proteins (FPs) as reporters in mycobacteriology started with the construction of green fluorescent protein (GFP) expressing *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG shortly after the development of bioluminescent strains [6]. Unlike the bioluminescent strains, fluorescent reporter strains do not require the addition of any exogenous substrate for detection. GFP reporter has been extensively used in mycobacterial research for transcriptional analyses and protein localization *in vitro* and *ex vivo* [7–10]. In the recent years, red-shifted variants of FPs (RFPs) have emerged as the FP reporters-of-choice for *ex vivo* and *in vivo* live imaging. Red-shifted excitation and fluorescence cause reduced autofluorescence and lower light-scattering, which make RFPs superior probes for *in vivo* imaging, particularly in deep tissues [11]. In the recent years RFPs and far-RFPs have been used successfully expressed in different mycobacteria [12,13]. MTB expressing RFP or far-RFP could also be visualized in live mouse models and their excised organs using *in vivo* imaging systems [12,14].

Supported by *in vitro* and *in vivo* findings, it is thought that a certain population of MTB enter into a dormant phase inside host granulomas [15,16]. Hypoxia or nutrient starvation *in vitro* induce dormancy [17–19] and hence is thought to play the same role inside hypoxic granulomas. It is also believed that the bacilli can persist, probably in dormant forms, at other sites away from lungs [20]. The dormant bacilli are non-replicating and hence are phenotypically tolerant to drugs that target actively dividing bacilli [21]. The dormant bacilli shut down most of the metabolic activity and remain viable in a quiescent state. This means that the bacilli exhibit minimal transcriptional activity and expresses only those genes required for survival in a certain microenvironment.

Hence, while using reporter strains of MTB, the choice of promoter used for expression of the reporter will decide the traceability of the bacilli in a microenvironment or an anatomical location. Since respiration is an indispensable process in both actively dividing and dormant bacilli, two promoters that drive the expression of two essential genes in the respiratory chain of MTB were tested. One is a promoter of *Rv1854c* that codes for type II NADH dehydrogenase/ndh, while the other is that of the adenosine triphosphate (ATP) synthase operon. There are two types of ndh in the MTB genome–a proton pumping, 14-subunit ndh-1 complex and an alternative, non-proton pumping, single-subunit ndh-2 [22]. Even though MTB possesses two copies of ndh-2 (ndh and ndhA), *ndh* is essential since insertional mutation of *ndh* was lethal while knock out of *ndhA* was tolerated [23]. Inactivating mutations in *ndh* of *M. smegmatis* too led to a thermosensitive lethal and auxotrophic phenotype [24]. Ndh not only acts as the electron donor in the anaerobic electron transport chain in MTB, but also is crucial for replenishing the pool of NAD+ during hypoxic non-replicating state [25]. However, even during aerobic growth, it has been proposed that type II NADH dehydrogenase is the sole NADH dehydrogenase enzyme in the MTB respiratory chain [22].

ATP synthesis *de novo* is vital for the viability of non-replicating MTB under hypoxic conditions [25]. This finding explains why the new anti-TB drug bedaquiline that targets the mycobacterial ATP synthase [26] is bactericidal to both replicating and non-replicating populations of MTB. However, it is also known that ATP synthase is down regulated during *in vitro* dormancy and inside phagosomes [19,27]. Hence the promoter element for ATP synthase operon was chosen to not only trace the bacilli but also to sensitively capture changes in its metabolic activity. In this study, it is demonstrated that both these promoters when coupled to a RFP help detect active as well as dormant mycobacteria in various model systems. The *ndh* promoter was more efficient than the ATP synthase promoter in detecting the corresponding reporter strains in the chronic phase of murine infection.

Materials and methods

Mycobacteria

Both wild type (WT) and RFP expressing MTB H37Rv were sub-cultured in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD) containing 10% oleic acid–albumin–dextrose-catalase (OADC) and 0.05% Tween 80, except that kanamycin (30 μg/ml) was added to the broth for the latter. WT and RFP strains of *M. smegmatis* mc^2155 were sub-cultured in Luria–Bertani broth containing 0.2% glycerol and 0.05% Tween 80 (LB-GT broth), except that kanamycin (30 μg/ml) was added to the broth for the latter.

Plasmids

Promoter less vectors pMV206 and pMV306 were kind gifts from Prof. WR Jacobs, Jr., Albert Einstein College of Medicine, New York, USA.

Construction of fluorescent strains

Turbo RFP 602 gene (Evrogen) was obtained codon optimized (for optimal expression in MTB) and synthesised from Integrated DNA Technologies, Coralville, IA, USA. The gene was cloned into *Eco*RI and *Hin*dIII restriction sites of pMV206 and