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An Inducible System for the Identification of Target Genes for a Regulator in Mycobacteria

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Abstract: We described principle and application of an inducible system for identification of target genes of a given mycobacterial protein with a regulatory function. This vector system, a promoter-probe vector, carries (i) promoterless *lacZ* as the reporter gene and (ii) the mycobacterial gene encoding the regulatory protein under the transcriptional control of the promoter of highly inducible mycobacterial gene encoding acetamidase. A promoter library of *M.tuberculosis* is constructed in this vector upstream of the *lacZ* gene. It is then possible to screen for those promoters that are responsive to the presence of the regulatory protein by inducing the expression of the regulatory gene. The presence of *lacZ* permits the screening of the promoters based on simple blue-white selection. This system is specifically designed for those regulatory genes of *M.tuberculosis* which are associated with virulence and thus are absent from *M.smegmatis* (the non-pathogenic, saprophytic species of mycobacteria), although in principle, modifications can be incorporated in the selection scheme to make it applicable for the identification of target promoter(s) of any regulatory gene of mycobacterial origin. This strategy will be helpful in quick identification of targets for the development of anti-tubercular drugs and in alleviating some of the stumbling blocks faced by the investigators working in the area of molecular genetics of *M.tuberculosis*.

Key words: gene regulation, plasmid vectors, inducible promoter

INTRODUCTION

Mycobacterial infections especially tuberculosis, compounded by the problems of AIDS and multidrug resistance, have emerged as a cause of grave global concern. A large number of laboratories in the world today have focused their attention on the study of biology and genetics of mycobacteria with the multifaceted aim of developing new drugs, elucidation of mechanism(s) of drug resistance and pathogenesis of *M.tuberculosis*, development of efficient diagnostic methods and new vaccines against tuberculosis. One of the key issues, which remains unresolved, pertains to the identification of genes which are exclusively present in *M. tuberculosis* and are involved in the establishment of the disease. During the last decade, useful information has been gathered about some of the genes involved in the pathogenesis of *M. tuberculosis,* which are present only in the members of *M.tuberculosis* complex and absent from non-pathogenic species such as *M.smegmatis.* However, verification of their function has been difficult specially due to lack of simple and foolproof methods for knocking out genes in *M.tuberculosis*. Another difficulty in the study of molecular biological aspects of mycobacteria has been the dearth of understanding about the control of gene expression. Unavailability of a tightly regulatable system for mycobacteria represents one of the caveats in gene expression studies. One such gene encoding the enzyme acetamidase (a 47 kDa protein) was identified from *M.smegmatis* [12] and its upstream region was characterized [13]. This gene exhibits a very high induction of expression in the presence of the inducer substrate acetamide, although only negligible amount of acetamidase could be detected in the absence of acetamide upon immunoblot analysis [13]. Similar results were obtained earlier by using *lux* gene encoding bacterial luciferase under the acetamidase gene promoter [7]. Here we describe the principle of this unique system from mycobacteria to identify the targets of a regulatory protein.

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MATERIALS AND METHODS

Bacterial strains and culture conditions: *M. smegmatis* LR222 was grown in Middlebrook (MB) 7H9 (for broth) and 7H11 (for plates) supplemented with 0.2% Tween-80. *Escherichia coli* DH5 α was grown in Luria-Bertani (LB) broth. Bacteria were cultured at 37°C with shaking at 200 rpm and whenever appropriate, antibiotics were added at the following concentrations: ampicillin (50 µg/ml), kanamycin (25 µg/ml).

Construction of pAK2 vector: For the construction of pAK2 vector, the acetamidase gene was excised from the plasmid pURR21 (a kind gift from Dr. T. Parish, Institute of Cell and Molecular Science, London, UK). As shown in Fig.1, the *Bgl*II-*Xba*I fragment containing the ∅10 promoter of T7 phage in pT7.2 was replaced with the *Bam*HI-*Sph*I fragment from the plasmid pURR21 containing the acetamidase gene promoter (after end repairing both the fragments) and the clone with the correct orientation of the promoter fragment was selected to result in pAK1. The gene encoding resistance to ampicillin was then excised out from pAK1 by digestion with *Dra*I and the remaining fragment was cloned in pSD5B vector [11] digested with *Nhe*I and *Dra*I (to excise the *E.coli* p15A origin of DNA replication) to obtain pAK2.

RESULTS

The VirS protein from *M. tuberculosis*, a putative regulator of pathogenesis of mycobacteria, was identified in our laboratory [8]. This protein has sequence and structural similarities with VirF protein of *Shigella*, VirFy protein of *Yersinia* and Cfad, Rns and FapR proteins of enterotoxigenic *E.coli* which in their respective systems bind to the promoter regions of their target structural genes and control a range of functions such as entry of the organism into the host cell, its survival in the anti-microbial environment of macrophages and spread into the neighbouring cells [8, 9]. The *virS* gene encoding the 38 kDa protein is present exclusively in the members of the *M.tuberculosis* complex [9]. One of the promoters upregulated by the VirS protein transcribes mymA operon [14,15]. In order to investigate the precise role of the VirS protein in *M.tuberculosis*, as a long-term goal, one of our approaches is to identify other promoters regulated by the VirS protein and subsequently study the functions of the associated genes and their protein products. To this end, we have designed a vector as described in Fig. 1. Thus, this vector contains the *virS* gene under the control of inducible acetamidase promoter and a promoterless *lacZ* gene of *E.coli* in addition to the origins of DNA replication from mycobacteria and *E.coli* and gene encoding kanamycin resistance for selection of transformants.

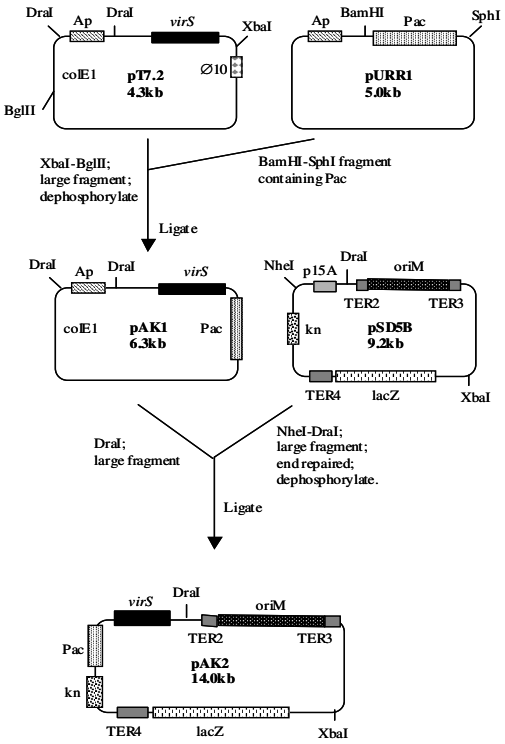

Fig.1: Construction of the vector pAK2 for identification of promoters regulated by the VirS protein of *M.tuberculosis*. *Pac* represents the upstream region encompassing the promoter of the inducible gene coding for acetamidase from *M.smegmatis*. *kn* codes for the gene for resistance to kanamycin for selection of transformants. ColEI and oriM are the origins of DNA replication from *E.coli* and mycobacteria, respectively. The *virS* gene coding for the VirS protein is placed under the transcriptional control of *Pac*. *Xba* I restriction site for cloning of promoter fragments is located upstream to the promoterless *lacZ* gene of *E.coli*. The transcriptional terminators are designated as TER2, TER3 and TER4, which represent the rrnBT1, the synthetic tryptophan terminator of *E.coli* and the *E.coli* phage fd terminator, respectively.

Strategy for the selection of target genes: The strategy for selection of the target genes subjected to regulation by the *virS* gene product by using the vector pAK2 is shown in Fig.2.

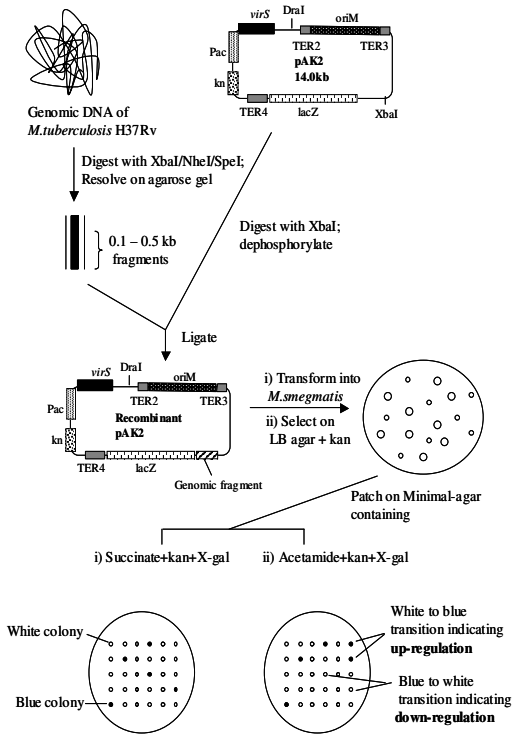

Fig.2: Schematic representation of the strategy for selection of promoters of the target genes of the VirS protein of *M.tuberculosis* by using the inducible mycobacterial acetamidase promoter in the vector pAK2.

M.tuberculosis genomic DNA is digested with either *Xba*I or *Spe*I or *Nhe*I or combination of these enzymes that generate overhangs compatible with *Xba*I. DNA fragments in the size range of 100-500 bp are cloned in the *Xba*I promoter cloning site upstream of the *lacZ* gene in pAK2 vector to construct a promoter library. The ligated products are electroporated into *M.smegmatis* used as the host and the transformants are selected on 7H11 plates containing kanamycin (25 μg/ml). Kanamycin resistant colonies thus obtained are patched under the non-inducing conditions with succinate (2 g l^{-1} final concentration) as a carbon source on minimal-agar plates (minimal medium [6] containing 2 ml l^{-1} trace elements [10], kanamycin and 1.5% agar) containing X-gal (40 μg/ml) for blue-white selection of colonies. In addition, all the colonies are patched on similar plates but containing acetamide (2 g l^{-1}) as a source of carbon instead of succinate thereby effecting induction of the acetamidase promoter followed by induction of the *virS* gene as a consequence. Any colony that turns blue from initial white or *vice versa* is scored to contain the *M.tuberculosis* promoter fragment which is regulated by the VirS protein. With this simple and rapid screening, all those promoters that are either up or down-regulated by the VirS protein can be identified. These promoters can be sequenced and their corresponding genes can be identified by analysing the sequence of *M.tuberculosis* genome. Fig. 3 depicts the results of immunoblot analysis to confirm the induction of the *virS* gene in minimal medium containing acetamide using *M.smegmatis* transformed with pAK2 and not in the cultures grown in medium containing succinate as carbon-source.

Fig.3: Immunoblot analysis for induction of the 38K gene expression in *M.smegmatis* transformed with pAK2. Transformants were grown in 10 ml of minimal medium (4) containing 2 ml l^{-1} trace elements (7), kanamycin (25 μg ml^{-1}) and succinate or acetamide (2 g l^{-1}) as carbon sources to A_{600nm} of 0.8. Cells were harvested, suspended in one-tenth culture volume of 0.1M Tris.Cl pH 7.6 containing 1 mM PMSF and sonicated. 10 μg of each of the cell-free extracts was resolved on a 10% SDS-polyacrylamide gel and total protein was transferred to nitrocellulose membrane. The expression of the *virS* gene was confirmed by immunoblot analysis using antisera to the VirS protein as indicated by an arrow. Lanes : cell-free extracts of cultures grown in minimal medium containing succinate (lane 1) and acetamide (lane 2).

DISCUSSION

This inducible system represents a powerful tool for the identification of target genes for a regulatory protein involved in the pathogenesis of *M.tuberculosis* as being used for the VirS regulatory protein in our studies [unpublished results]. One could think of using *E.coli* as a host for this purpose, an organism which is faster to grow, easier to work with and where elegant systems for such studies are known. However, there are two serious caveats (i) a number of mycobacterial proteins are now being shown to carry mycobacteria specific post-translational modifications such as glycosylation[2,5] which could be important for the optimal functioning of these proteins (ii) most of the mycobacterial promoters do not function well in *E.coli*[1,3]. The screening method described here overcomes both of these obstacles by making use of a mycobacterial inducible promoter and a mycobacterial species as a host thus providing native environment for the screening purpose.

It is possible to modify this system by using a two-plasmid system instead of one-plasmid system as described here. The module comprising of the acetamidase gene promoter fused to the regulatory gene can be stably integrated in the *M.smegmatis* genome by using integration proficient vectors such as pDK20[4] and the promoter library constructed in pSD5B [11] can be screened in a fashion similar to that described above for pAK2 in the recombinant *M.smegmatis* strain.

This strategy can be extended to identify the targets of any regulatory gene with function other than that in pathogenesis provided the regulator in question is not endogenously expressed in *M.smegmatis*. In the event of the regulatory gene being present in *M.smegmatis*, the chromosomal copy of the gene from *M.smegmatis* can be deleted, as homologous recombination in *M.smegmatis* can be carried out rather easily unlike in the case of *M.tuberculosis* and the mutant strain can be used for the screening purposes. In view of the limitations and challenges faced by the investigators working with *M.tuberculosis*, we hope that this system will be useful in overcoming some of the problems in the study of mycobacterial molecular genetics and provide the basis for the development of similarly useful systems for the study of gene regulation in other organisms.

CONCLUSION

This report describes a simple and powerful, blue-white selection based, strategy for the identification of target genes of virulence associated regulatory proteins of *M. tuberculosis* by employing the highly inducible promoter of acetamidase gene. This strategy will be very useful in quick identification of new targets for the development of anti-tubercular drugs.

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