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Regulated Expression Systems for Mycobacteria and Their Applications

Dirk Schnappinger[1] and **Sabine Ehrt**[2]

[1]Department of Microbiology and Immunology, Weill Medical College, and Program in Molecular Biology, Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10065

[2]Department of Microbiology and Immunology, Weill Medical College, and Program in Immunology and Microbial Pathogenesis, Weill Graduate School of Medical Sciences of Cornell University, New York, NY 10065

Abstract

For bacterial model organisms like *Escherichia coli* and *Bacillus subtilis* genetic tools to experimentally manipulate the activity of individual genes existed for decades. But for genetically less tractable yet medically important bacteria such as *M. tuberculosis* such tools have rarely been available. More recently several groups developed genetic switches that function efficiently in *M. tuberculosis* and other mycobacteria. Together these systems utilize six different transcription factors, eight different regulated promoters, and three different regulatory principles. Here we describe their design features, review their main applications, and discuss advantages and disadvantages of regulating transcription, translation, or protein stability for controlling gene activities in bacteria.

Introduction

Genetic elements that enable specific and quantitative control over the activity of individual genes are irreplaceable components of the modern genetic toolbox. They facilitate not only the purification of proteins for biochemical, structural, or immunological studies but can also be applied to improve our understanding of *in vivo* gene functions. Until recently only one such tool was available for use in mycobacteria and its applicability in slow growing mycobacteria was limited. But during the last decade no less than a dozen new systems have been developed. Here we review the design, components, and regulatory mechanisms of the different systems and discuss their main applications.

Genetic switches for controlling gene expression in mycobacteria

The acetamidase system (Figure 1a)

During growth with short aliphatic amides (e.g. acetamide) as the primary carbon source *M. smegmatis* induces expression of the acetamidase encoded by *amiE* (1–3). The regulatory elements of this gene were utilized to generate the first inducible expression system for

For correspondence: dis2003@med.cornell.edu or sae2004@med.cornell.edu; Tel.: 212 746-2994 or 212 746-3788.

mycobacteria (4). The system proved valuable for the production of mycobacterial antigens (4, 5) and enabled the first silencing studies of essential genes (e.g. *whmD* and *dnaA*) in *M. smegmatis* (6, 7). But genetic instability limited the use of this system in *M. tuberculosis* (8) and its complexity – regulation of *amiE* involves three regulators (AmiC, AmiD, and AmiA) (9, 10) – prevented its optimization. While the acetamidase system has been largely replaced by other tools, especially in *M. tuberculosis*, a derivative, which incorporated the T7 RNA polymerase (RNAP), remains one of the best tools available to achieve high-level overexpression of a protein in *M. smegmatis* (11).

TetON and TetOFF (Figure 1b and 1c)

Tetracycline (tc) resistance of many bacteria is caused by efflux pumps whose expression is – due to the fitness defect the pumps cause in the absence of drug pressure – tightly regulated. This regulation is mediated by a single repressor protein, the tc repressor (TetR), which specifically binds two operators ($tetO_1$ and $tetO_2$) in the promoter that drives transcription of the efflux pump (12). In the complex with TetR the tet promoter (P_{tet}) is masked from access by RNAP and initiation of transcription is inhibited. When tc enters the bacterial cell it binds to TetR and induces transcription of the efflux pump before the drug can inhibit the ribosome. This sensitivity towards low drug concentrations is due to the remarkable affinity of TetR to tetracyclines, which is up to 10^5-fold higher than the ribosome's affinity to tetracyclines (13).

In 2005, three groups independently reported TetR-controlled expression systems for mycobacteria (14–16). The systems shared the same basic design but differed in the origin of their regulatory components: The TetRs were derived either from the *Corynebacterium glutamicum* resistance determinant TetZ (14, 17) or the *E. coli* transposon Tn*10* (15, 16); the regulated promoters were either also from TetZ (14, 17), derived from the *B. subtilis* xyl promoter (16), or constructed by inserting *tetO*s into a mycobacterial promoter (15). All three systems can be induced with low concentrations of tetracyclines in a dose-dependent manner with the preferred inducer either being tc (for the TetZ-derived systems) or anhydrotetracycline (atc) (for the two systems that utilize the Tn*10* TetR). Because tc/atc has to be added to induce expression we refer to these systems as "TetON" systems.

The Tn*10* TetR has been the subject of many mechanistic analyses. In a screening strain that proved particularly useful, TetR controlled expression of β–galactosidase while the lac repressor (LacI) and transcription of *galK*, encoding galactokinase, were repressed by LacI. This allowed to identify amino acids required for binding of TetR to *tetO* (mutations in these amino acids led to β–galactosidase positive and galactokinase negative colonies without atc) (18) or for induction of TetR by tetracyclines (19). A mutagenesis originally performed for the latter purpose also identified the first TetR that only bound *tetO* in complex with tc. Such reverse TetRs were later adapted for use in mycobacteria to construct a "TetOFF" switch in which transcription of the target gene is turned off by the addition of atc (20, 21). Optimization of these repressors for use in mycobacteria included adapting the guanine-cytosine (GC) content of the encoding genes to that of mycobacteria, which increased TetR expression and also led to an improved TetON system (21). TetON and TetOFF have been used by several groups to analyze gene functions in *M. smegmatis* and *M. tuberculosis*

(Table 1). They also provide the basis for some of the other regulatory expression systems developed more recently (22–24) and a tunable coexpression system to analyze protein-protein interactions (25).

AraC and LacI (Figure 1d and 1e)

Leakiness, i.e. expression without inducer, is a limitation of many regulated expression systems. One of the most tightly regulated *E. coli* expression systems is the pBAD system (26). Its promoter, P_{BAD}, is controlled by two regulators: AraC, which represses the promoter without arabinose and activates it in its presence, and the catabolite activator protein, CAP, which acts as a second activating factor (27). Activation of P_{BAD} by CAP increases with the intracellular cAMP concentration. In *E. coli*, activity of P_{BAD} without arabinose can thus be reduced by adding glucose to the growth medium because glucose decreases cAMP levels in this species. Unfortunately, P_{BAD} does not function in *M. smegmatis* as it does in *E. coli* (28) and there is no apparent advantage that pBAD has over the other systems developed for mycobacteria. Tight regulation of P_{BAD} in *E. coli* depends not only on protein-DNA interactions but also on direct protein-protein interactions of AraC and CAP with RNAP as well as low levels of cAMP. It therefore would be difficult to optimize the pBAD system for use in mycobacteria.

Other frequently used *E. coli* expression systems depend on promoters that are repressed by LacI and induced with IPTG (29). Two studies demonstrated the value of LacI for regulating gene expression in mycobacteria. The first applied LacI to repress a promoter recognized by the T7 RNA polymerase (30); the second inserted a lac operator (*lacO*) downstream of a mycobacterial promoter to impose susceptibility to repression by LacI (31). For both systems little expression was measured without IPTG, but no follow up studies or applications have been published and their value for broader studies remains to be determined.

NitR (Figure 1f)

The saprophytic actinomycete *Rhodococcus rhodochrous* encodes several nitrilases, which detoxify nitriles by hydrolyzing them into their carboxylic acid and ammonia (32). Under optimal conditions *R. rhodochrous* J1 increases nitrilases expression up to ~3,000-fold, which results in the nitrilase encoded by *nitA* accounting for ~35% of total soluble protein (32, 33). This drastic overexpression is achieved via a positive feedback loop controlled by NitR, a member of the AraC family of transcriptional regulators. The molecular mechanism by which NitR acts has not been investigated in detail. But NitR alone is sufficient to mediate induction of P_{nitA} and its own promoter in other bacterial species, most likely functioning as a direct activator of transcription initiation (34, 35). In *M. smegmatis* NitR strongly activated transcription after addition of either ε-caprolactam or isovaleronitrile whereas in *M. tuberculosis* only isovaleronitrile was effective (36). The positive feedback loop that is generated by NitR's activation of its own promoter distinguishes this system from all other expression systems available for mycobacteria and has three consequences: (i) Induction is strong, (ii) on a single-cell level the switch is either ON or OFF, and (iii) intermediate inducer concentrations create two sub-populations, one that has NitR-controlled gene expression turned fully ON and one that is still in the OFF-state. In contrast,

intermediate concentrations of atc partially activate the TetON system so that the average expression level of most cells increases to levels between the OFF and fully induced states (36).

PipON and Tet/PipOFF (Figure 1g and 1h)

Pristinamycin belongs to the streptogramin group of antibiotics, which consist of at least two structurally unrelated but synergistically acting molecules. In the case of pristinamycin, these two molecules are pristinamycin I and pristinamycin II, both of which inhibit bacterial ribosomes (37). Resistance of *Streptomyces pristinaespiralis* to pristinamycin is due to the pristinamycin resistance gene, *ptr*, which encodes a multidrug efflux pump (38). The *ptr* promoter, P_{ptr}, is repressed by the transcription factor Pip and can be activated with pristinamycin I, pristinamycin II, and several other antibiotics (39, 40). Pip belongs to the TetR family of transcription factors and binds to three sites in P_{ptr}, two of which overlap with the promoters −35 and −10 hexamers (40). P_{ptr} is a strong promoter in *M. smegmatis* and *M. tuberculosis*, can be efficiently repressed by Pip and induced with low concentrations of PI. As a consequence the PipON system has an excellent regulatory range (41).

The Pip system was also adapted to confer repression upon addition of atc. In contrast to the TetOFF system, which utilizes a reverse TetR, in Tet/PipOFF Pip is placed under the control of wt TetR so that atc increases expression of Pip. The target gene is located downstream of P_{ptr} and thus repressed as a consequence of the increased Pip expression. When desired, PI can be used to overcome the repression caused by atc (24). A system with a similar regulatory circuit had placed TetR under the control of the acetamidase system (42).

Controlled proteolysis (Figure 1i and 1j)

Bacterial regulatory circuits often rely on posttranscriptional modifications, which include controlled degradation, to achieve rapid inactivation of a protein. In fact, posttranscriptional modification is crucial to quickly inactivate proteins with a long half live as their abundances only change slowly even after transcription and translation have stopped (43). The recognition sites of bacterial proteases include C-terminal degradation tags (44). One such tag gets added to proteins in a process called *trans*-translation and is encoded by the small stable RNA *ssrA* (45). In *E. coli ssrA*-tagged proteins are degraded by several proteases including ClpXP, which directly binds to the tag's C-terminal amino acids (46). Affinity of ClpXP to the *ssrA*-tag is increased by the adaptor protein SspB, which binds both the tag's N-terminus and ClpX (47, 48). Proteins containing the *ssrA*-derived DAS+4-tag depend on the tethering of ClpXP to the tag by SspB. As a consequence they are only degraded when SspB is expressed (Figure 1i). This SspB-dependency is due to mutations that change the tags C-terminal amino acids from Leu-Ala-Ala to Asp-Ala-Ser (hence the "DAS") and weaken the direct interaction with ClpX and an insertion of four amino acids (hence the "+4") that facilitates simultaneous binding of SspB and ClpX (49). Interestingly, SspB is also capable of delivering DAS+4-tagged proteins to ClpXP in bacteria that do not themselves encode an SspB homolog (50). This provided the mechanistic basis for one type of gene silencing tool that utilizes proteolysis to deplete proteins in mycobacteria (23). A second such tool was developed by placing the *ssrA*-tag upstream of a protecting peptide that can be removed by a site-specific protease derived from HIV-2 (labeled as *hivP* in

Figure 1j). The resulting tag was named inducible degradation (ID) tag (22). In both systems degradation of the tagged protein is induced with atc, which turns on expression of either SspB or the HIV-2 derived protease.

The theophylline riboswitch (Figure 1k)

Riboswitches are regulatory elements, in which binding of a small molecule to an RNA aptamer results in a change in gene expression (51). They are entirely RNA-encoded and do not require any trans-factors besides the aptamer-binding ligand, which can simplify transferring functional riboswitches from one species to another (52). The riboswitch adapted for use in mycobacteria is induced by theophylline (53), a methylxanthine drug used to treat pulmonary diseases (54). In the absence of theophylline the switch forms a secondary structure that masks the ribosome binding site (RBS) and thus prevents translation. Binding of theophylline stabilizes an alternative secondary structure, which liberates the RBS and induces translation of the regulated mRNA.

Common and distinctive features of the different regulatory systems and strategies

The ideal system for manipulating gene expression would (i) be completely silent under repressing conditions, (ii) provide a large (i.e. >1,000-fold) regulatory range that can be adjusted in a dose-responsive manner with a small-molecule that has no direct effects other than controlling the targeted gene, (iii) not interfere with the target's native regulation under inducing conditions, (iv) leave the protein sequence unchanged, and (v) allow rapid gene induction and protein depletion in growing and non-replicating bacteria in vitro and during infections of host cells and animals. Not surprisingly, such a system has yet to be developed. But the available systems approach these features to different degrees.

Regulatory range

The range of regulated expression systems can be easily assessed using reporter gene assays. It is often calculated by dividing the reporter activity under inducing conditions by that measured under maximally repressing conditions. For most systems this has been achieved using either GFP, β–galactosidase, or luciferase as reporter. A regulatory range of >100-fold was measured for several systems (i.e. two of the TetON systems, the NitR system, PipON, and SspB-mediated proteolysis) with the largest range having been reported for PipON (Table 1).

Leakiness

Identifying expression systems that permit moderate expression without inducer is straightforward and can be achieved using the same reporter gene assays used to measure their regulatory range. However, none of the reporter assays that have been used to characterize mycobacterial expression systems approach single-molecule sensitivity. Lack of detectable reporter activity under repressing conditions, which has been reported for several systems, can therefore not provide proof of complete repression. In fact, all mycobacterial expression systems most likely permit some low level of expression without inducer. Whether or not this leakiness interferes with the goals of an experiment is difficult to predict

and depends on the question that is being addressed and the gene under investigation. However, when necessary, the leakiness of an expression system can be reduced by decreasing the efficiency with which the targeted mRNA is translated (55).

Dose-responsiveness

All systems besides the one regulated by NitR have either been demonstrated to be dose-responsive or are likely to be dose-responsive in the sense that intermediate concentrations of the inducer or corepressor result in intermediate expression levels within most of the bacteria. Lack of dose-responsiveness of the NitR system comes at the benefit of achieving very high expression in the induced state.

Invasiveness

Controlling a gene's expression is not possible without changing at least its promoter, the 5′ non-coding end of its mRNA, or the 3′-end of its open reading frame. An alteration of the promoter is required to allow for transcriptional regulation, the incorporation of the riboswitch changes the mRNA's translation initiation sequence and its 5′-end, and the gene's 3′-end and the C-terminus of the encoded protein need to be changed to achieve controlled proteolysis. Fortunately, these modifications have little impact on the function of many genes but any one of them can prevent complementation of a particular mutant. Strategies that rely on controlling transcription and/or translation have the advantage to leave the open reading frame of the targeted gene unchanged. Controlled proteolysis on the other hand can leave a target's native regulation of transcription and translation intact. The theophylline riboswitch can also be used in combination with a gene's native promoter and does not require changes of the regulated protein. Riboswitches can thus provide the least invasive strategy to artificially control gene expression in bacteria.

Regulation during infections

Providing control over *M. tuberculosis* gene expression during infections is a key ability of expression systems designed for this pathogen. Evidence that this can be achieved during macrophage infections has been obtained for TetON/OFF, PipON, Tet/PipON and the systems controlled by LacI or NitR. However, only for TetON/OFF have experiments been reported that demonstrated efficient regulation can be achieved in animal models (55–60).

Applications

Ectopic expression

One motivation for the construction of the acetamidase system was to enable purification of *Mtb* or *M. leprae* proteins from a fast-growing mycobacterial host, which was expected to yield proteins better suited for structural and immunological studies than those expressed in *E. coil* (4). The need for a mycobacterial expression host is supported by the finding that >50% of all *Mtb* proteins can either not be efficiently produced in *E. coli* or accumulate as insoluble inclusion bodies (61). For these proteins *M. smegmatis* can be a superior expression host because its codon usage is very similar to that of pathogenic mycobacteria, which facilitates high level expression of proteins encoded by GC-rich mRNAs. Furthermore, proteins that accumulate as insoluble inclusion bodies in *E. coli* can – at least

in some cases – be expressed as soluble proteins in *M. smegmatis* (61). Purification of polyhistidine-tagged recombinant proteins from *M. smegmatis* can be complicated by contamination with co-purified GroEL1, but this can be avoided by using an *M. smegmatis* strain, in which the histidine-rich C-terminus of GroEL1 has been removed (62).

More recently ectopic expression was also used to analyze gene functions in *M. smegmatis* and *M. tuberculosis*. Many of these studies focused on type I toxin-antitoxin (TA) modules. These modules consist of two proteins that are often encoded by bicistronic operons wherein the 5′-gene encodes the antitoxin and the 3′-gene the toxin. As long as expression of the TA module continues, the toxin is bound and neutralized by its cognate antitoxin. Once expression stops, the inherently instable antitoxin is degraded leading to release and activation of the toxin. The *M. tuberculosis* genome encodes 88 putative TA modules, many of which are conserved within the *M. tuberculosis* complex yet absent from other mycobacteria (63). For many of these putative toxins, inducible overexpression was used to confirm that they are indeed functional toxins capable of arresting growth of *M. smegmatis* and/or *M. tuberculosis* (Table 1). This growth arrest generally does not occur upon simultaneous overexpression of the cognate antitoxin, i.e. the antitoxin encoded within the same TA module, but is not relieved by overexpression of other antitoxins (63). Another informative application has been to combine ectopic overexpression of DNA binding proteins witch chromatin immunoprecipitation (ChIP). This was first demonstrated in experiments that defined the *in vivo* binding sites of SigA and several alternative sigma factors (64). Recently, this approach has been extended to define the binding sites of many DNA binding proteins in *M. tuberculosis* (65).

Gene silencing

Controlled gene silencing allows studying a gene's *in vivo* function under a variety of conditions even if the gene is required for growth. One conceptionally attractive strategy to conditionally inactivate a gene is to destabilize and prevent translation of its mRNA with an antisense RNA. This strategy was first applied to reduce expression of AhpC in *M. bovis* (66) and has since been used to inactivate several genes in *M. smegmatis* and *M. tuberculosis* (Table 1). One study in particular reported striking phenotypes for antisense-mediated gene silencing in several *M. tuberculosis* conditional knockdown (cKD) mutants (31). Attempts to silence different essential genes in *M. smegmatis* or *M. tuberculosis* with antisense RNAs of varying lengths in our own unpublished work have unfortunately all failed. The reasons for this failure are unclear to us and might be technical in nature. However, it is noteworthy that several research groups resorted to gene silencing approaches that are more complicated and time consuming than antisense mediated gene inactivation. Antisense mediated gene silencing thus likely failed frequently, which suggests either that expression of only a few genes is susceptible to antisense inhibition or that some of the factors important for the functionality of an antisense RNA remain to be identified.

An alternative to expressing antisense RNAs is to exchange the targeted gene's promoter so that its transcription can be regulated directly. Promoter exchange can be achieved *in situ*, i.e. in the native chromosomal location, either by integrating a suicide plasmid immediately upstream of the targeted gene (15), by selecting for a double-crossover event that deletes the

native promoter and replaces it with a regulated promoter (59), or by transposon insertion (67, 68). These strategies have been applied in many cases and most cKD mutants of *M. tuberculosis* or *M. smegmatis* published to date employed direct transcriptional repression (Table 1). Obtaining phenotypically well-regulated conditional knockdown mutants can, however, be challenging, especially for genes that only need to be expressed at a low level to be functional. In *M. tuberculosis*, *bioA* represents such a gene, whose mRNA is of low abundance during logarithmic growth (69, 70). It encodes the biotin biosynthetic enzyme 7,8-diaminopelargonic acid synthase, which is dispensable with extracellular biotin but essential for growth when biotin cannot be scavenged from the environment. The first *BioA* TetON mutant constructed with the Tn*10*-derived TetON system overexpressed BioA protein ~10-fold compared to wt *M. tuberculosis* (55). Removal of inducer decreased BioA expression by ~100-fold yet only mildly reduced growth. In its original form the Tn*10*-derived TetON system contains a strong P_{tet} located upstream of a strong translation initiation site. Strength of the promoter and the translational initiation site were likely both responsible for overexpression of BioA. It was unclear if decreasing promoter strength would sufficiently reduce *bioA* transcription without inducer; but weaker translational initiation sites were expected to decrease both BioA overexpression with inducer and leaky expression without inducer. Accordingly, cKD mutants containing a weak translational initiation signal upstream of the *bioA* open reading frame reproduced the phenotype of a *bioA* deletion and only grew with inducer when growth depended on biotin synthesis (55). In our hands, this strategy of minimizing the phenotypic consequences of transcriptional leakiness with weak translation initiation signals has been successful for several other targets (unpublished data) and is generally useful to improve the efficiency of transcriptional gene silencing.

Another elegant use of direct transcriptional silencing is its combination with transposon mutagenesis. This depends on a transposon carrying a regulated promoter at one end in the outward-facing direction and allows identifying well-regulated mutants based on their growth phenotypes (68).

cKD mutants that utilize transcriptional repression can be constructed by in situ promoter exchange, and similarly cKD mutants that utilize controlled proteolysis can be generated by modifying a gene's 3′-end within its native location in the genome. This strategy has so far only been applied to the construction of cKD mutants in *M. smegmatis*, but shown good success in this species (22, 23, 71, 72). Nevertheless, for some targets depletion by controlled proteolysis was insufficient to produce the expected phenotypic consequences. For example, inactivation by controlled proteolysis of dihydrofolate reductase (DHFR) or alanine racemase (Alr), which are both essential for growth, depleted these enzymes by more than 97% but only modestly decreased growth of *M. smegmatis* (22).

Controlling gene expression during infections

Mutations that attenuate *M. tuberculosis* can cause *growth in vivo* (*giv*), *severe giv* (*sgiv*), and *persistence* (*per*) phenotypes in mice (73). *Giv* mutants replicate substantially less than wt, and *sgiv* mutants do not grow at all in mice whereas *per* mutants replicate normally but fail to persist. Genes required for growth and persistence, i.e. genes whose inactivation

causes *sgiv* and *per* phenotypes can only be identified by conditional inactivation. The mycobacterial Tet systems helped demonstrate that the three *sgiv* genes *bioA*, *pckA*, encoding phosphenolpyruvate carboxy kinase and *icl*, which encodes isocitrate lyase, are required by *M. tuberculosis* not only to grow in mice and establish an infection, but also to persist during the chronic phase of the infection (55, 57, 58). A cKD mutant of the *in vitro* essential CarD revealed that *M. tuberculosis* depends on this transcriptional regulator for replication and persistence in mice (60). Similarly, 4′-Phosphopantetheinyl transferase PptT was shown to be required for the replication and survival of *M. tuberculosis* during the acute and chronic phases of infection in mice and helped validate these enzymes as a potential new drug target (56). The appearance of revertants, which are unresponsive to TetR mediated transcriptional control, can complicate the analysis of essential genes *in vitro* and *in vivo* (reference (60) and our unpublished observations). A careful analysis of the bacterial population expressing the regulated gene under investigation is therefore necessary for conclusive data interpretation.

Target-based whole cell screens

The application of regulated expression systems that can impact drug development most directly is their use in target-based whole cell screens. Such screens employ mutants in which expression of the target protein has been decreased to the extent that it limits the growth rate, which increases sensitivity towards small molecule inhibitors of that protein. This principle was initially established with *Staphylococcus aureus* strains, which were engineered to express growth-limiting amounts of FabF and showed an increased susceptibility to FabF-inhibitors but not to other antibiotics (74). Whole cell screens against this FabF-underexpressor identified platencin and platensimycin, the founding members of a new class of fatty acid biosynthesis inhibitors with broad-spectrum activity against Gram-positive bacteria (75–78). *M. tuberculosis* mutants expressing lower than wt levels of PanC, LysA, Icl1, or LepB have recently been constructed and also show target-specific changes in their susceptibility to different small molecule inhibitors (79, 80). Whole cell screens with these strains promise to identify new inhibitors of pantothenate synthase, diaminopimelate decarboxylase, isocitrate lyase, and the type I signal peptidase, respectively.

Conclusions and future perspectives

When the first edition of this book was published the only regulated expression system available was the acetamide system. Since then a dozen new regulatory systems have been developed that together utilize six different transcription factors (TetR, revTetR, AraC, LacI, NitR, and Pip) and eight different regulated promoters (P_{BAD}, P_{T7Lac}, P_{trc}*lacO*, P_{nitA}, P_{ptr}, and three different P_{tet} promoters). They were applied not only to facilitate purification of correctly folded proteins but also to study mycobacterial gene functions within their native hosts either by ectopic expression or conditional inactivation. By now several mycobacterial expression systems function so efficiently that their use in most applications is straightforward. However, the isolation of phenotypically well-regulated cKD mutants remains challenging, irrespectively of the regulatory system one chooses for mutant construction. Reducing expression with antisense RNAs has been successful for some genes, but failed to silence at least as many. This is unfortunate, because antisense-based gene

silencing does not require manipulation of the host chromosome by homologous recombination. It would thus become the most straightforward approach to generate cKD mutants if its success rate could be improved.

Direct transcriptional silencing was often but not always successful. Due to the inherent leakiness of most regulated promoters direct transcriptional silencing is most inefficient for genes whose products are only needed in low amounts. The opposite is likely true for controlled proteolysis because highly expressed proteins will burden the host's proteolytic machinery more than proteins expressed at a lower level. That transcriptional silencing and controlled proteolysis can both fail to produce phenotypically well-regulate cKD is essentially a consequence of their limited dynamic range, which spans only 2 orders of magnitude. In contrast, *M. tuberculosis* gene expression, as measured by RNA sequencing, spans at least 4 to 5 orders of magnitude (69, 70). One of the main remaining challenges in the development of regulated expression systems for mycobacteria is thus to expand their dynamic range. In ongoing work we observed that this can be achieved by combining transcriptional repression with controlled proteolysis. This strategy of combining existing regulatory systems that differ in their mechanism of regulation could be further extended. For example, it should be possible to combine the theophylline riboswitch with any of the transcriptional regulation systems to reduce their effective leakiness yet still allow high level expression when necessary.

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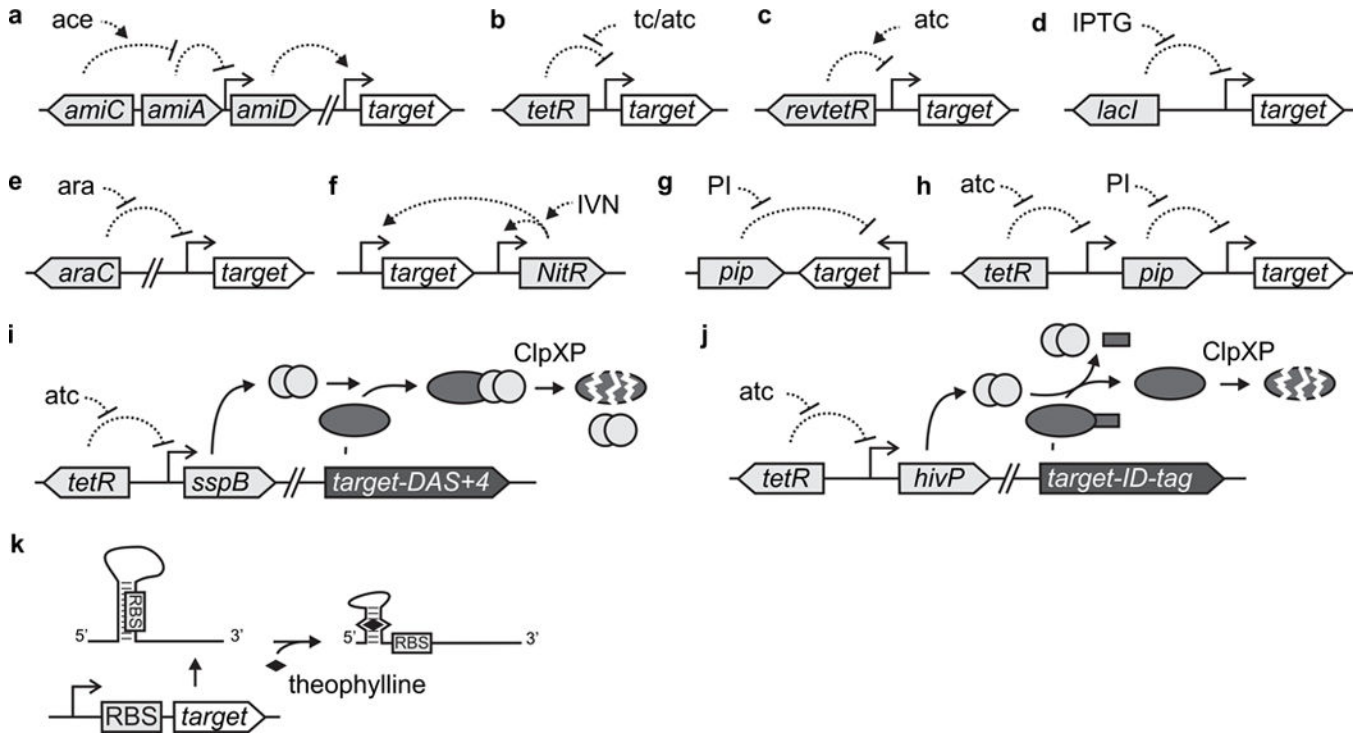

Figure 1. Regulatory systems for mycobacteria

The transcriptional regulatory systems are shown in (**a**) to (**h**), the two controlled proteolysis systems in (**i**) and (**j**), and the theophylline riboswitch in (**k**). Dotted lines ending in a perpendicular line indicate negative regulatory interactions; dotted lines ending in an arrow represent positive regulatory interactions. Ace, acetamide; tc/atc, tetracycline / anhydrotetracycline; IPTG, isopropyl β-D-1-thiogalactopyranoside; ara, arabinose; IVN, isovaleronitrile.

Table 1

Regulated expression systems for mycobacteria and examples of their applications.

Expression System	Components	Regulatory range	Applications
A. Regulation of transcription			
Acetamidase	AmiC, AmiD, AmiA, promoters Pc, P1, P1, and P3 (9, 10)	~80-fold induction of *M. leprae* 35 kD protein in *M. smegmatis* (4) 22-fold induction of FtsZ in *M. smegmatis* (81)	Ectopic expression of mycobacterial antigens (4, 5), PknA/B (82, 83), and toxin-antitoxin proteins (63) in *M. smegmatis* Silencing of *whiB2* (*whmD*) (6), *wag31* (82, 83), *kasA* (84), *inhA* (84), *dnaA* (7) in *M. smegmatis*
	Acetamidase inducible T7 RNAP	Not reported.	Overexpression of putative drug targets in *M. smegmatis* (11)
TetON	TetR and P_{tet} from TetZ (14)	230-fold induction of luciferase activity in *M. smegmatis* (14, 17) 13-fold induction of luciferase activity in *M. tuberculosis* (14) 21-fold induction of luciferase activity in *M. bovis* BCG (14)	Ectopic expression of Ms2173 (85) and toxin-antitoxin proteins (86–89) in *M. smegmatis* Silencing of *ftsZ* (14), *wag31* (82, 83) *clpC1*, *pknB*, *msmeg_2694* (90), *parA* (91) and *glmM* (92) in *M. smegmatis*. Silencing of *clpC1* (90), *ppk1* (93), *ppk2* (94) and *dosR* (95) in *M. tuberculosis*.
	Tn*10* TetR; P_{myc1}*tetO* (15)	170-fold induction of GFP activity in *M. smegmatis* (14) 160-fold induction of β–galactosidase activity in *M. tuberculosis* (14)	Ectopic expression of PhoP (96), DosR (97), various sigma factors (64), various transcription factors (65), I-SceI (98, 99) PzaA/PncA (100), BirA (101), Pbp1 (102), EspR (103) and toxin-antitoxin proteins (86, 104–107) in *M. smegmatis*, *M. bovis* BCG, and/or *M. tuberculosis* Silencing of *ftsZ* (14), *secA1* (20, 108), *pptT* (109) *ripA* (110) *pbp1* (102) *ppm1* (111) *carD* (60) *msmeg_3935* (112) *clpP* (71) in *M. smegmatis*. Silencing of *icl* (58), *rv3671c* (58), *prcBA* (58, 59) *pptT* (56, 109) *espA* (113) *bioA* (55) fba (114) *esx*-3 (115) *carD* (60) *pckA* (57), *clpP* (71), panC (79), lysA (79) *dfrA* (116) in *M. tuberculosis*.
	Tn*10* TetR; P_{xyl}*tetO* (16)	~10-fold induction of GFP activity in *M. smegmatis* and *M. tuberculosis* (16)	Silencing of *trpD* (16), *dprE1* (117), *clpP1* (117), *fadD32*, *glnA1* (117), *glnE* (117), *pknL* (117), *regX3* (117), *senX3* (117) in *M. tuberculosis*
TetOFF	Reverse TetR; P_{myc1}*tetO* (20, 21)	50-fold repression of β–galactosidase activity in *M. smegmatis* (21) 10-fold repression of β–galactosidase activity in *M. bovis* BCG (21)	Silencing of *secA1* (20) in *M. smegmatis*. Silencing of *prcBA* (59) *panC* (79), *lysA* (79), *icl* (79) in *M. tuberculosis*.
pBAD	AraC, P_{BAD} (28)	~3-fold induction of β–galactosidase activity in *M. smegmatis* (28)	Ectopic expression of Rv1991c (28)
LacI	LacI, P_{T7Lac}, T7 RNAP (30)	40-fold induction of GFP protein in *M. tuberculosis* (30)	Identification of metabolically active *M. tuberculosis* in macrophages (30)
	LacI, P_{trc}lacO	30-fold induction of β–galactosidase activity in *M. smegmatis* (31)	Silencing of *ftsZ*, *gyrA*, and *gyrB* in *M. smegmatis* (31). Silencing of *gyrA*, *gyrB*, *inhA*, *embR*, *rpoB*, *rpoC*, *rplJ*, *rpsL*, and *ilvB* in *M. tuberculosis* (31).
NitR	NitR, P_{nitA} (36)	>100-fold induction of XylE activity in *M. smegmatis* (36) ~100-fold induction of XylE activity in *M. tuberculosis* (36)	
PipON	Pip, P_{ptr} (41)	52-fold induction of β–galactosidase activity in *M. smegmatis* (41). 450-fold induction of β–galactosidase in *M. tuberculosis* (41).	Silencing of *fadD32* (41) and *pknB* (41) *ftsK* (68), *glf* (68), *infB* (68), *leuA* (68), *metC* (68), *rne* (68), *rv0883c* (68), *rv1478* (68), *rv2050* (68), *rv2204c* (68), *secY* (68), *tuf* (68) in *M. tuberculosis*

Expression System	Components	Regulatory range	Applications
TetPipOFF	Tn*10* TetR, Pip, P_{myc1}*tetO*, P_{ptr} (24)	~60-fold repression of β–galactosidase in *M. smegmatis* and *M. tuberculosis* (24)	Silencing of *ftsZ* (24) in *M. smegmatis*. Silencing of fadD32 (118) in M. abscessus. Silencing of *fadD32* (24), *eccB5* (119), *eccC5* (119) esx-3 (120) in *M. tuberculosis*.
B. Regulation of protein stability			
DAS+4-tag	Tn*10* TetR, P_{myc1}*tetO*, SspB	36-fold, 250-fold repression of GFP and luciferase activity in *M. smegmatis*, respectively (23) 7-fold repression of GFP activity in *M. tuberculosis* (23).	Depletion of RpoB in *M. smegmatis* (23)
ID-tag	Tn*10* TetR, P_{myc1}*tetO*, HIV2 derived protease	~80-fold repression of GFP activity in *M. smegmatis* (22) >30-fold depletion of Alr protein; ~5-fold depletion of RpoB protein in *M. smegmatis* (22)	Depletion of Alr (22), DHFR (22), InhA (22), GyrA (22), KasA (22), RpoB (22), and FhaA (72) in *M. smegmatis*
C. Repression of translation			
Theophyllin	Riboswitch (53)	65-fold and 89-fold induction of GFP and β–galactosidase activities in *M. smegmatis* (53) 8-fold induction of GFP activity in *M. tuberculosis* (53)	Silencing of *katG* in *M. smegmatis* (53)