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Random Inducible Controlled Expression (RICE) for Identification of Mycobacterial Virulence Genes

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SUMMARY

We have developed Random Inducible Controlled Expression (RICE), a high throughput genetic approach to identify regulated virulence pathways in pathogenic mycobacteria. RICE allows expression of bacterial genes under conditions where they are normally off, e.g. under laboratory growth conditions, via the use of an inducible or constitutive promoter as well as gene dosage effects due to the presence of the gene on a plasmid. Mycobacterial genomic DNA can be digested to yield random fragments for cloning into a suicide expression vector downstream of a mycobacterial promoter or with their own promoter on a replicating plasmid increasing expression by gene dosage effects. The plasmid DNA is normally amplified in *E. coli* and delivered into mycobacteria to select for recombinants or plasmid transformants. The resulting library is then directly screened for enhanced host cell interactions in functional assays that evaluate the efficiency of adherence, entry and replication inside host cells. This approach has resulted in identification of several virulence factors from pathogenic mycobacteria. Our analysis of one such locus identified by RICE, the mycobacterial enhanced entry locus (*mel2*), found that the genes present facilitate bacterial persistence inside the host by protecting the pathogen against oxidative damage. Thus, we have developed a genetic strategy that offers several advantages: (i) it allows identification of bacterial genetic elements that have a direct role during host-pathogen interactions (ii) it can be used to identify virulence factors in a broad range of pathogens and (iii) it can reveal genes that are only induced at specific stages of infection.

Pathogens have evolved tight regulatory networks to induce and repress genes involved in virulence to conserve energy and this regulation is clearly evident in *Mycobacterium tuberculosis*^{1–3}. When a pathogen invades a host cell, an initial stress response is induced and the genes necessary for bacterial survival and replication are turned on. This is usually followed by induction of genes necessary for persistence such as microaerophilic and starvation responses and repression of genes involved in general metabolism and replication. Taken together, these observations suggest that pathogens would only rarely constitutively express virulence genes. In mycobacteria, evidence that virulence genes are regulated includes observations that both the growth phase and intracellular growth have an impact on mycobacterial entry into host cells. Mycobacteria harvested from early to late logarithmic phase enter macrophages nearly five-fold more efficiently than lag phase cultures^{4, 5}. Furthermore, when grown in monocytes or amoebae mycobacteria enter eukaryotic cells nearly ten-fold more efficiently than media grown bacterial populations. These data indicate

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that at least some of the genes involved in mycobacterial virulence are regulated and repressed under standard laboratory growth conditions.

Several approaches have been developed to identify virulence genes in *M. tuberculosis* and other pathogens. In vivo expression technology (IVET) identifies genes that are specifically induced in vivo using a promoter trap strategy⁶. Selective capture of transcribed sequences (SCOTS) identifies bacterial transcripts with greater abundance in vivo relative to in vitro growth⁷. Multi-pronged approaches such as signature tagged mutagenesis (STM), transposon site hybridization (TRASH), differential fluorescence induction (DFI) and more recently deep sequencing technologies have also been used to identify mutants with reduced virulence^{8–10}. The central theme of these approaches is to identify genes that are differentially expressed inside the host. However, they do not reveal the specific role of these virulence genes during host pathogen interactions. We have developed a genetic approach called Random Inducible Controlled Expression (RICE) to identify previously unknown pathways by which *M. tuberculosis* regulates virulence (Figure 1). We have successfully used RICE in mycobacteria^{4,5} and *Legionella pneumophila*¹¹ to identify genes involved in adherence, entry and subsequent survival inside macrophages. This strategy can be easily adapted to elucidate virulence determinants that are regulated at specific stages of infection from nearly any bacterial pathogen.

RICE requires the ability to experimentally control expression of all genes in the genome of the pathogen, either through the use of a promoter of known strength and regulatory properties randomly integrated into the chromosome or through gene dosage effects resulting from the presence of a genomic library on a plasmid. These approaches allow for either inducible or constitutive gene expression of all genes in the genome under standard laboratory conditions where virulence genes are normally repressed. A shuttle cosmid, pJDC16, was created that contains mycobacterial origin of replication from pAL5000 and a unique *Bam*HI site⁴. *M. marinum* chromosomal DNA was partially digested with *Sau*3AI resulting in genomic fragments of 40–50 kb. Fragments were dephosphorylated and ligated into cosmid pJDC16 at its unique *Bam*HI site. These were packaged in vivo in *E. coli* strain χ 2819 and the resulting cosmid carrying phage lysate was used to infect *E. coli* strain XL1-Blue. Kanamycin resistant colonies that arose after growth were pooled and the cosmid DNA prepared and transformed into *M. smegmatis*⁴ and *M. marinum*⁵. These recombinant mycobacterial strains carrying *M. marinum* genetic elements were used in cell entry assays. Several independent pools of *M. smegmatis* and individual clones of *M. marinum* cosmid libraries that represented the entire *M. marinum* genome were used to identify clones that conferred efficient adherence and entry into macrophages. As a result of these screens we have identified several new mycobacterial genes that are involved in host cell interactions and many of these are present in *M. tuberculosis*^{4,5} and have similar functions in *M. tuberculosis*^{12–14}. One of the potential limitations of RICE is that over expression in this manner might lead to toxicity for the bacteria, but this problem can be easily circumvented by conditional expression using an inducible promoter.

The characterization of *M. marinum* RICE libraries resulted in the identification of several virulence factors, designated mycobacterial enhanced entry loci (*mel*) and repressed entry loci (*mrl*)^{4,5,13}. While some *mel* loci are prevalent in all mycobacterial species, others, such as *mel2*, are restricted to *M. tuberculosis* and *M. marinum*. A mutation in the *M. marinum mel2* locus can be fully complemented by *M. tuberculosis mel2*, suggesting similarities in *mel2* function between these two species. Interestingly, genes present in *mel2* have significant amino acid similarity to bioluminescence genes from other bacterial species¹⁴. Bioluminescence genes have been suggested to play a role in defense against reactive oxygen species^{15–17}. Furthermore, it is recognized that *M. tuberculosis* undergoes

oxidative damage during latency¹⁸ and we have shown that *mel2* facilitates persistence of *M. tuberculosis* in mice by defending against reactive oxygen species¹².

We analyzed the amino acid sequence of MelF, encoded by the first open reading frame (ORF) in the *mel2* locus, using Conservative Domain Search in National Center of biotechnology (NCBI)¹⁹. We found that MelF contains an alkanesulfonate monooxygenase-like domain. This domain is found in two-component systems that catalyze the conversion of alkanesulfonates to the corresponding aldehyde and sulfite, similar to *E. coli* alkanesulfonate monooxygenase (SsuD)²⁰. The second component of this model, the NADPH-dependent FMN oxidoreductase (SsuE), has similarity to MelG, encoded by the second ORF in the *mel2* locus, which consists of multiple domains with flavin reductase-like motifs. The resulting working model, summarized in Figure 2, could explain the observed role of the *mel2* locus in resistance to oxidative damage and its similarity to the *lux* locus found in *Vibrio fischeri*^{12–14}. We expect to gain insight into the validity of this model through analysis of the biochemical and physiological roles of these proteins. We have now successfully overexpressed the ORFs within the *mel2* locus (Figure 3), the first step toward a better understanding of their biochemical activities. Because of their apparent role in persistence, a comprehensive understanding of the biochemical activities of these loci, including interactions between the different ORFs, could lead to rational strategies to block their activities and better manage tuberculosis latency.

In summary, we have developed a high throughput genetic approach designated Random Inducible Controlled Expression (RICE) that has allowed the identification of new pathways by which *M. tuberculosis* regulates virulence. We are currently using biochemical analyses to dissect the mechanisms of action for these unique virulence pathways.

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Fig. 1. Identification of regulated virulence genes by random inducible controlled expression (RICE). RICE libraries can be created by cloning genomic fragments from a pathogen of interest into suicide expression vectors. The resulting genomic library can then be used to randomly insert a promoter into the host chromosome via homologous recombination. The recombinant library can then be used in selective assays or as individual overexpressing clones to select for a desired function to identify genes that play a specific role in pathogenesis, regardless of their normal regulatory characteristics.

Fig. 2.

Working model for the functions of MelF and MelG. A proposed interaction model between MelF and MelG proteins encoded by the *mel2* locus, as predicted based on conserved domains and phenotypic characteristics of mutants constructed in *M. marinum* and *M. tuberculosis*. Oxidation of alkanesulfonates to the corresponding aldehyde would utilize a reactive oxygen species (ROS) molecule, explaining the increased susceptibility of *mel2* mutants to the host immune response.

Fig. 3. Overexpression of Mel proteins. Demonstration of overexpression of MelF with C-terminal His-tag (MelF-His) in *E. coli*. Total protein resolved on a 10% acrylamide SDS PAGE gel stained with Coomassie blue, Lane 1: un-induced *E. coli* BL21 (DE3) carrying MelF-His, lane 2: 1 mM IPTG induced *E. coli* BL21 (DE3) carrying MelF-His, lane 3: auto-induced (glucose/glycerol/lactose growth ²¹) *E. coli* BL21 (DE3) carrying MelF-His and lane 4: Protein Marker Precision Plus Standard (Bio-Rad). Arrow indicates the expected position of MelF-His, based on size markers.