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US-Japan Co-operative Tuberculosis and Leprosy Research Panel, 2003. Front Row: Hatsumi Taniguchi, Masamichi Goto, Masao Mitsuyama, Patrick Brennan and Toru Mori. Back Row: Kiyoshi Takatsu, Gail Jacobs, Tom Gillis, Gilla Kaplan, Christine Sizemore, Philip Hopewell and David McMurray.

ABSTRACTS

Spencer, J. S., Kim, H. J., Gonzalez-Juarerro, M., Marques, M. A. M., Williams, D. L., Sang-Nae Cho, and Brennan, P. J. Lessons learned from cloning, expression, and immunologic analysis of unique *Mycobacterium leprae* proteins.

With the recent completion and annotation of the genomes of *M. tuberculosis* and *M. leprae*, and additional sequence data from environmental mycobacteria (*M. avium*, *M. marinum*, and *M. smegmatis*), our ability to identify and test leprosy-specific antigens is much improved. Comparative genomic analysis of the *M. leprae* genome has identified 1604 open reading frames, as well as 1116 inactivated genes (pseudogenes), and up to 165 genes with no orthologue in *M. tuberculosis*. Through comparative genomic analysis of all mycobacterial databases, we have targeted 28 of these novel *M. leprae* genes for cloning and expression as recombinant proteins. This report characterizes our initial findings of five of these selected unique proteins, with a comparison of several other *M. leprae* proteins that have homologues in *M. tuberculosis*. The unique proteins tested were ML0008, ML0394, ML0126, ML1057, and ML2567, while proteins with homologues include the well-known 10 kDa GroES (ML0380), Ag85B (ML2028), ESAT-6 (ML0049) and CFP-10 (ML0050) proteins. After cloning the genes from *M. leprae* genomic DNA by PCR, all were produced as single recombinant proteins in *E. coli*. Our strategy was to produce polyclonal antisera against all of these single proteins, and then to use each antiserum to examine the relative amounts of the individual proteins in the native subcellular fractions of *M. leprae* (cytosol, membrane, and soluble cell wall antigens). In this way, we had previously shown the existence of native *M. leprae* ESAT-6 in the cell wall fraction in amounts significant enough to suggest that it could stimulate antibody or T cell responses in a natural infection. Indeed, several investigators have recently shown that leprosy patient PBMC respond well to both *M. leprae* ESAT-6 and CFP-10 proteins and peptides. Our initial analysis of the five unique *M. leprae* proteins by Western blot showed no detectable levels of any of these proteins in the native subcellular fractions. Nevertheless, by RT-PCR analysis of *M. leprae* cDNA isolated from two geographically different strains of *M. leprae* (Thai-53, originally isolated from a patient from Thailand, and strain 4089, originally isolated from a patient from Mexico), it appears that all of these genes have mRNA transcripts, and, thus, are potentially transcribed. However, further testing of these five recombinant proteins in *M. leprae*-sensitized guinea pigs did not reveal any detectable delayed type hypersensitivity (DTH) response. In addition, the proteins were examined by ELISA assay using leprosy patient sera (including sera from multibacillary lepromatous and paucibacillary tuberculoid patients). Responses to the unique antigens showed much lower responses overall, with the majority of the O.D. readings falling in the low to background level response range, consistent with the level of these antigens produced, while responses to well expressed antigens were much higher. It is possible that some of these novel proteins are just not that immunogenic. Alternatively, despite the lack of a significant antibody response, T cells from PBMC of leprosy patients may react better in IFN-γ assays to some of these unique proteins or their peptides, a possibility we are currently exploring. In light of our current findings, we should seriously question the strategy of choosing antigens for diagnostic purposes based on comparative genomics alone. It would appear from these results that some genes unique to *M. leprae*, although capable of generating a transcript, are not expressed in sufficient quantities in the bacillus itself to induce a meaningful immune response, and hence should not be pursued for diagnostic purposes. Alternative strategies for the selection of promising antigens will be discussed.

Groathouse, N. A., Rivoire, B., Sang-Nae. Cho, Brennan, P. J., and Vissa, V. D. Polymorphisms in Short Tandem Repeat Sequences of *Mycobacterium leprae* allow for the Epidemiological Characterization of Strains.

Up until now, the characterization of strain and lineage of *Mycobacterium leprae* isolates has been a great obstacle to studying the epidemiology of leprosy. In this post-genome era, the availability of a complete DNA sequence for *M. leprae* has provided an opportunity to target and analyze specific sites within the genome. Our approach for molecular typing of *M. leprae* includes the detection of variable number of short tandem repeat (STR) sequences, in different isolates. The specific goal of this research is to identify several polymorphic sites and combine them into a multiplex PCR-based molecular typing tool, which could be used on clinical biopsy samples collected in endemic parts of the world. We hope to develop a product (technique and reagents) that is portable, easy to apply and analyze, and inexpensive.

The first step, localization of regions of the genome within the sequenced Tamil Nadu (TN) strain containing a succession of single, di- and tri-nucleotide repeats, was accomplished by utilizing the search pattern program of the Leproma database (http://genolist.pasteur.fr/leproma/). A panel of 31 distinct sites in non-coding regions that contained 5 or more repeat units was identified. From these, several primary sites were selected. Primer sets were designed to flank the individual STR sites and elicit PCR products of incremental length to facilitate multiplexing in the future. A total of six *M. leprae* DNA sources (armadillo derived human clinical isolates) were used to search for polymorphisms by comparing the STR lengths. The PCR products were cloned to enable sequence confirmation of the entire amplified fragment, including the repeat region.

So far, we have confirmed repeat length polymorphisms in four of the DNA sources, and in all but one STR sequence. One of the AT repeat regions, showed as few as 25 bp and as many as 37 bp within the STR sequence of the various DNA sources when compared to the 37 bp sequence reported in the TN strain. Likewise, a different AT repeat sequence varied from 20 to 30 bp in the different DNA isolates while it is at 34 bp in the TN strain. A multi-C repeat sequence that is 20 bp in the TN strain ranged from 8 to 14 bp in the *M. leprae* isolates. Alternatively, a CG repeat sequence was found to have the same length as the TN strain, in all isolates tested.

Our findings clearly indicate that polymorphisms are present at various sites in the genomes of different strains of *M. leprae*, and consequently could be developed to track their lineage. Further emphasis will be placed on the use of fluorescent 5′ labeled primers for amplification, detection and fragment length analysis of the multiplexed products from the six DNA isolates on sequencing gels to improve throughput. The next phase will be expansion of the analysis to some of the remaining selected loci, followed by inclusion of a larger collection of DNA isolates. The final goal is to produce a fingerprinting system for *M. leprae* obtained from clinical isolates and to provide a tool for studying other aspects of leprosy such as transmission, virulence, drug resistance and relapse.

Williams, D. L. Gene Expression and Stability of mRNA in *Mycobacterium leprae*.

Evaluation of gene expression in *Mycobacterium leprae* under different experimental conditions should provide important insight into the physiology and therefore, the life-style of this noncultivable mycobacterium. Recently, we have identified mRNA transcripts for 194/200 genes analyzed by RT-PCR and the presence of polyadenylated mRNAs in 15/15 transcripts analyzed by oligodT priming of total RNA. Since polyadenylation in bacteria leads to mRNA stabilization under some conditions and degradation under other conditions, polyadenylation of mRNA in *M. leprae* may not be a useful indicator of mRNA stability. Nothing is known about the stability of mRNA in this slow growing pathogen. The purpose of the current study was to determine the relative stability of selected polyadenylated mRNAs in *M. leprae* after termination of transcription. To accomplish this, a suspension of mouse footpad-derived *M. leprae* in 7H12 medium was killed by subjecting bacteria to 5×10^6 rads of γ-radiation for 4 hr followed by rifampin (8 μg/ml final concentration) treatment to terminate mRNA transcription. RNA was purified from untreated bacteria (live) and the from dead bacteria stored at 33°C for 24, 48, 72 hr and

1 week post treatment. The expression of five *M. leprae* genes from various gene families, previously found to be transcribed in the mouse foot pad (*rpoB, gyrA, folP1, hsp18,* and *sodA*), was determined using semiquantitative RT-PCR. Appropriate controls for DNA contamination were included for each template analyzed. The 16S rRNA PCR product from each template was used to normalize these data for template variations. Results demonstrated that transcripts for all genes studied were detected in the live bacteria for the entire experiment. In addition, transcripts for *rpoB, folP1*, and *hsp18* were observed up to one week post death, although transcript levels were lower than that of the untreated controls and progressively decreased with time. Transcripts for *gyrA* were observed up to 48 hr post treatment, although transcript levels were lower than those found in the live bacteria. Transcripts for *sodA* were not observed in the 24 hr treatment cDNA indicating that this mRNA is less stable than the rest of the mRNAs studied. These results demonstrated that the relative stability of the selected polyadenylated mRNAs varied suggesting that polyadenylation in *M. leprae* can not be used as an indicator of mRNA stability. Therefore, it is recommended that mRNA stability analysis be conducted on genes of interest prior to designing gene expression experiments for *M. leprae* because down regulation of the expression of a gene may not be detected in the background of a stable transcript encoded by that gene. In addition, the relatively short stability of the *sodA* transcripts suggests that further studies should be conducted to determine if mRNA is a suitable target for the development of rapid real time RT-PCR assay for the viability of *M. leprae*.

Truman, R., Fontes, A., Suffys, P., Gillis, T. Characterization of VNTR Polymorphisms in *Mycobacterium leprae*.

Differentiating mycobacteria at the subspecies (strain) level based on genomic diversity can be useful in understanding their evolution and for discriminating relationships between different clinical cases or laboratory strains. Early attempts to define strain variants of *M. leprae* have been of limited value because *M. leprae* appeared to show limited heterogeneity at the phenotypic and genomic levels. Insertion sequences are not abundant in *M. leprae* and, therefore, are not expected to provide sufficient intrusion into the genome to create variation useful for developing a strain typing system. Similarly, neither restriction fragment length polymorphism analysis (RFLP) nor 16S rDNA sequencing has identified diversity among *M. leprae* isolates from patients or natural animal hosts. More recently, various repeating elements have been shown to be effective in genotyping other highly conserved bacterial species. The completed sequence of the *M. leprae* genome reveals a number of such elements and some limited diversity already has been reported among a few clinical specimen within these repetitive sequences.

In Japan, limited variation in a 6 bp repeat in the *rpoT* gene was found among isolates from one region but it was absent among isolates in other locales. Somewhat greater diversity has been observed among *M. leprae* derived from a number of patient samples in the Philippines, where polymorphism was observed ranging from 10 to 37 copy numbers of a TTC (GAA) triplet repeat occurring in an intergenic segment downstream of pseudogene. Along with a few other repeating elements of unknown diversity, the significance of these variable number tandem repeat (VNTR) markers in *M. leprae* and their utility for epidemiological or clinical study has not yet been described.

We examined the diversity of GAA (same as TTC repeat described by Shin, *et al.*) repeats along with 3 other VNTR markers using a battery of clinical samples and *M. leprae* reference strains derived from patients and wild animals in different geographic regions. To help assess the suitability of these VNTR markers for laboratory and community based studies, we also examined the stability of the reference strain VNTR genotype with passage in different animals under varied conditions and in different tissues.

Lahiri, R., Randhawa, B., and Krahenbuhl, J. Further definition of viability of *Mycobacterium leprae* as a research resource.

A rapid and reliable method to compare viability between two suspensions of *Mycobacterium leprae* is needed. We have shown previously that a radiorespirometry method, which assesses the metabolic activity of *M. leprae*, compares well with the mouse footpad assay. In this study, we tested a two-color (Syto9 and Propidium iodide) fluorescence assay to determine if a rapid direct count viability staining can be applied to *M. leprae*. A variety of experimental conditions were employed to assess this "viability stain." We also applied both radiorespirometry and viability staining on extracellular and intracellular *M. leprae* treated with different known antimycobacterial drugs to assess the reliability of these two methods taken together, in screening anti-leprosy drugs. We used Rifampin, Clofazamine, Dapsone, Ofloxacin, and Minocycline, on axenic and intracellular cultures of *M. leprae*. We found that the intracellular model of drug testing was more efficient in detecting anti bacterial effect of drugs in *M. leprae* than axenic cultures.

Matsuoka, M., Liangfen, Z., and Budiawan, T. Analysis of leprosy transmission based on genotyping.

Mycobacterium leprae isolates were divided into two groups by the polymorphism in the *rpoT* gene. Geographic distribution of the *rpoT* genotype of *Mycobacterium leprae* in Latin American countries was investigated in connection with human prehistoric migration. All *M. leprae* isolates from Peru and Paraguay showed three tandem repeats of 6 bp. On the contrary, 25 out of 27 isolates from Mexico revealed four tandem repeats. It was assumed that the leprosy was carried into these countries by different groups of ancient Mongoloids migrated to Latin America at different periods.

Genotyping by TTC repeat polymorphism was applied for epidemiological analysis of leprosy transmission. *M. leprae* on the nasal mucous membrane of villagers showed variety of TTC repeat numbers. *M. leprae* genotype of four leprosy cases in two houses was examined. *M. leprae* isolates from a couple of the father and a son showed the same TTC genotype but the father and the son in another house were infected by different *M. leprae* distinguished from TTC repeat. The findings suggested the transmission of the bacilli might be from various infectious sources, though it is generally believed the main infectious source is multi-bacillary patients and household contact with such patient has the highest risk of being infected. Infectious sources other than multi-bacillary patients in the household would be important for leprosy control especially in endemic countries. The results indicated the existence of the infectious source other than patients in the household.

Ohyama, H., Takeuchi, K., Yamada, H., Uemura, Y., and Matsushita, S. SNPs on IL-12 receptor gene associated with the susceptibility to leprosy.

Activated T cells from lepromatous leprosy patients are likely to produce low amounts of IFN-gamma even in the presence of IL-12. The objective of this study is to reveal the low productivity of IFN-gamma in leprosy patients from an immunogenetical viewpoint. The polymorphism of 5′ flanking regions of both *IFNG* and *IL-12RB2* were determined to compare the allele frequencies between patients and healthy donors, using direct sequencing technique. The results of the study are as follows. i) No polymorphism was detected in the promoter region of *IFNG*. ii) Several SNPs were detected in the 5′ flanking region of *IL-12RB2*, and SNPs located at the positions, –1035, –1023, –650 and –464 were more frequently detected in LL patients than in TT patients.

These results suggest that the polymorphism in the 5′ flanking region of *IL12RB2* may be implicated to determine disease form of leprosy.