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Original Article

Sequence Homology and Expression Profile of Genes Associated with DNA Repair Pathways in *Mycobacterium leprae*

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Abstract

Background: Survival of *Mycobacterium leprae*, the causative bacteria for leprosy, in the human host is dependent to an extent on the ways in which its genome integrity is retained. DNA repair mechanisms protect bacterial DNA from damage induced by various stress factors. The current study is aimed at understanding the sequence and functional annotation of DNA repair genes in *M. leprae*. **Methods: T**he genome of *M. leprae* was annotated using sequence alignment tools to identify DNA repair genes that have homologs in *Mycobacterium tuberculosis* and *Escherichia coli*. A set of 96 genes known to be involved in DNA repair mechanisms in *E. coli* and Mycobacteriaceae were chosen as a reference. Among these, 61 were identified in *M. leprae* based on sequence similarity and domain architecture. The 61 were classified into 36 characterized gene products (59%), 11 hypothetical proteins (18%), and 14 pseudogenes (23%). All these genes have homologs in *M. tuberculosis* and 49 (80.32%) in *E. coli*. A set of 12 genes which are absent in *E. coli* were present in *M. leprae* and in Mycobacteriaceae. These 61 genes were further investigated for their expression profiles in the whole transcriptome microarray data of *M. leprae* which was obtained from the signal intensities of 60bp probes, tiling the entire genome with 10bp overlaps. **Results:** It was noted that transcripts corresponding to all the 61 genes were identified in the transcriptome data with varying expression levels ranging from 0.18 to 2.47 fold (normalized with *16SrRNA*). The mRNA expression levels of a representative set of seven genes (four annotated and three hypothetical protein coding genes) were analyzed using quantitative Polymerase Chain Reaction (qPCR) assays with RNA extracted from skin biopsies of 10 newly diagnosed, untreated leprosy cases. It was noted that RNA expression levels were higher for genes involved in homologous recombination whereas the genes with a low level of expression are involved in the direct repair pathway. **Conclusion:** This study provided preliminary information on the potential DNA repair pathways that are extant in *M. leprae* and the associated genes.

Keywords: DNA repair, gene expression, homology, *Mycobacterium leprae,* phylogeny, transcriptome

Introduction

Stability and integrity of genetic information is crucial to cell survival and multiplication. Both prokaryotes and eukaryotes contain a repertoire of DNA repair pathways that are crucial to protecting the DNA from a myriad of harming errors which can be caused by various external and intracellular factors. Environmental agents such as chemicals, ultraviolet light and ionizing radiation, as well as errors in DNA metabolism, challenge the chemical structure and stability of the genome. These etiological factors lead to a variety of alterations in the normal DNA structure such as single- and double-strand breaks, chemically modified bases, abasic sites, inter- and intra-strand cross-links, and base-pairing mismatches. Given this diversity of threats and their effects, it is not surprising that there is a corresponding diversity in DNA repair pathways.[1] The diversity in functions and complexity of DNA repair pathways is better understood by comparing the mechanisms of action of each of the pathways. Most of what is thought for bacterial DNA repair mechanisms is derived from research in *Escherichia coli* (*E.coli*). However, genome sequencing has revealed many

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genes with unknown capabilities, and clear variations improve questions about the ubiquity of similar DNA repair pathways in the bacterial kingdom. For instance, many species of bacteria, including *E. coli*, lack an end joining pathway and depend on non-homologous recombination to repair double stranded breaks and alternatively on non-homologous end joining mechanisms (NHEJ).[2] Proteins associated with NHEJ were identified in a number of bacteria, some of which include *Bacillus subtilis*, *Mycobacterium tuberculosis*, *Mycobacterium smegmatis*,[3-6] and *Mycobacterium marinum*.[7] Bacteria utilize a remarkably compact version of NHEJ wherein all the required activities are contained in only two proteins: a *Ku* homodimer and a multifunctional ligase/polymerase/nuclease *LigD*.[8]

Originating from the family of Mycobacteriaceae, the genus Mycobacteria consists of pathogens known to cause serious diseases in humans, including tuberculosis and leprosy. The etiological agent of leprosy is *Mycobacterium leprae*. *This bacteria* has never been successfully grown on an artificial cell culture medium.[9] Instead, it has been grown in mouse foot pads and in armadillos. Armadillos develop infection and manifest disease. *M. leprae* also has the longest doubling time of 14 days.[10] Due to the absence of an axenic culture medium for propagation, studying cellular processes, especially those belonging to DNA repair pathways is often challenging. In general, the genes involved in DNA repair mechanisms are a part of the core metabolism and Possess similarity with *E. coli* and other Mycobacterial genomes, however intriguing minor differences suggest biological diversity in bacterial responses to DNA damage.

In this study, the genes in *M. leprae* that possess a probable role in DNA repair pathways, were identified and annotated using computational and laboratory tools. Initially, a bioinformatics approach was employed to analyze and describe the open reading frames (ORFs) in the genome of *M. leprae*, that are potentially related to DNA repair mechanisms. *M. leprae* specific homologues and orthologs of genes corresponding to DNA repair pathways in *E. coli* and *M. tuberculosis* were identified from the public databases. Most of the genes indicated a range of similarity and identity with orthologs in the genome of *M. tuberculosis*. However, *M. leprae* does not possess genes of the typical mismatch repair (MMR) system that are found in most of the other bacteria. Although *M. leprae* and *E. coli* belong to separate phylogenetic groups, many of their DNA repair genes possess substantial similarity. However, some of the vital DNA Repair genes that are present in *E. coli,* are absent in *M. leprae*.[11] Conversely, some of the functionally related genes that are present in *M. leprae*, are absent in *E. coli.*

Methods

Sequence annotation to identify DNA repair genes in *M. leprae* genome

The putative ORFs of *M. leprae* were compared with known DNA repair related genes obtained from public databases using the "BlastP" and DELTA-Blast search over Genbank non-redundant (nr) database of proteins. In a few precise cases, potential DNA repair genes in *M. leprae* genome were identified both by sequence similarity searches (using seed sequence orthologs from other organisms) and keyword searches. The candidate genes that are associated with DNA repair pathways are therefore confirmed by sequence similarity searches and domain analysis using CDD Blast on a Conserved Domain Database (National Centre for Biotechnology Information (NCBI)).

Sequence phylogeny analysis

Sequence similarities and evolutionary relatedness of all the probable DNA repair genes in *M. leprae* which are identified by above methods, were further analyzed by searching for orthologous and paralogous sequences in KEGG SSDB database using Smith–Waterman (SW) scoring matrix.[12] Phylogenetic trees were generated for a group of hypothetical protein orthologs and paralogs present in Mycobacteriaceae family. Protein sequences were aligned using "MUSCLE" (multiple sequence alignment program)[13] and manually adjusted with "Bio-Edit"(http://www.mbio.ncsu.edu/bioedit/bioedit.html). The maximum likelihood phylogenies with 100 bootstrap replicates were performed with PhyML[14] using the "Phylogeny.fr."[15]

Identification of ribosome binding sites and promoters

Nucleotide sequences of putative promoter regions for selected hypothetical proteins were obtained from publicly available databases. For all open-reading frames, 200 nucleotides upstream of the translation initiation site were considered while mapping promoters. Ribosome binding sites (RBS) and promoter sequences were predicted for a common motif by DNA alignments using MUSCLE.[13]

Insights from whole transcriptome microarray experiments

To determine the activity of the DNA repair genes, expression levels of these genes were analyzed in the transcriptome of *M. leprae* (whole RNA extracted from human skin biopsies of newly diagnosed untreated leprosy cases) using unpublished data on whole transcriptome experiments conducted by Chaitanya *et al*. (Schieffelin Institute of Health Research and Leprosy Center, Karigiri) (GEO dataset: GSE85948 private series). Differential gene expressions in terms of signal intensities of the DNA repair genes in the microarray experiment were normalized with that of *16SrRNA*, which is most commonly used housekeeping gene to measure the basal level of mRNA expressions in prokaryotes.[16,17] The median intensity value of *16SrRNA* as noted from the experiments is 8.051386 and this value was used to calculate the expression folds.

Quantitative polymerase chain reaction (qPCR) experiments

Source of Mycobacterium leprae RNA

M. leprae RNA was obtained from the skin biopsies of active leprosy patients. A total of 10 newly diagnosed untreated leprosy cases from the Dermatology Outpatient Department

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of "Schieffelin Institute of Health–Research and Leprosy Centre", Karigiri, Tamil Nadu, India, were enrolled in the study following the institutional ethical guidelines. An informed and written consent for participation was obtained from all the subjects before enrolling in the study, following the ethical guidelines as laid down by the Indian Council of Medical Research. All the procedures conducted in the study were in accordance with the guidelines of the institutional ethical committee and with the ethical standards as laid down in the 1964 declaration of Helsinki and its later amendments or comparable ethical standards. The excisional skin biopsy samples were collected in RNA later (Catalog No: R0901, Sigma-Aldrich) in aseptic conditions, by a clinician and were sent to Molecular Biology laboratory for RNA extraction and quantitative polymerase chain reaction (qPCR) experiments.

RNA extraction

RNA extraction was performed using RNeasy Blood and Tissue Kit (Catalog No: 74104; Qiagen Inc., USA) according to manufacturer's protocol. Aseptically, 2 mm × 2 mm size skin tissues were cut from the actual biopsy sample and were minced/grinded thoroughly using manual glass homogenizer. Alternatively, the tissues (up to 30 mg) were disrupted in Buffer RLT and homogenized using Tissuelyser LT (Catalog No.: 69980, Qiagen Inc., USA). Ethanol was added to the lysate to promote selective binding of RNA to the RNeasy membranes. The sample was then applied to the RNeasy Mini spin column. The contaminants were washed twice and high-quality RNA was eluted in RNase-free water. Genomic DNA contamination was removed by performing DNase treatment (Catalog No.: EN0521, Thermo Fischer Scientific). To rule out the presence of DNA contamination in the RNA samples, a PCR was set up for *16SrRNA* gene of *M. leprae* directly from the RNA samples without reverse transcription reaction. P2 and P3 primers as reported earlier[18] were used in the PCR amplifications. complementary DNA (cDNA) was constructed from 1 µg of total RNA from each of the sample using high-capacity cDNA reverse transcription kit (Catalog No.: 4368814, Applied Biosystems).

Quantitative polymerase chain reaction

Based on the expression levels of the DNA repair genes identified from the transcriptome data, genes corresponding to a set of 4 highly expressed and annotated proteins and 3 highly expressed hypothetical proteins were selected for qPCR experiments to determine/confirm the expression levels. cDNA corresponding to these 10 transcripts was amplified on a Rotor Gene-Q qPCR machine (Qiagen Inc., USA, Serial Number: R0414139) using respective primers [Table 1] and by following reaction conditions. A volume of 20 µl reaction mix containing 10 µl of QuantiNova SYBR Green PCR Master Mix (Qiagen, Cat No: 208054), 0.25 µM (0.5 µl) concentration of each of the forward and reverse primers for respective genes, 7 µl of nuclease free distilled water and 2 µl of cDNA (containing approximately 200 ng) were cycled in Rotor-Gene Q. Cycling conditions include one cycle of hold at 95°C for 2 min (initial denaturation and activation of enzyme) followed by 40 cycle of 95°C for 10 s, annealing at 60°C for 15 s and elongation at 72°C for 20 s. Fluorescence was acquired on green channel during the annealing step. This was followed by a melting step which involves an increase in temperature from 72°C to 95°C at a rate of 1°C/s. Melting curve analysis was performed to determine the integrity of the amplification and to rule out primer-dimer formation.

Analysis of quantitative polymerase chain reaction data

The mRNA expression levels were normalized using *16SrRNA* as a reference. The threshold fluorescence values were normalized to those of *16SrRNA* threshold fluorescence (Ct) values. The mRNA expression levels were calculated after determining the primer efficacy for all the targets using Pfaffl Method[16] by a standard curve with a 7-fold dilution of *M. leprae* DNA from 500 pg/reaction to 7.813 pg/reaction. Melting curve analysis was performed to determine the integrity of the amplification and to rule out primer-dimer formation. PCR for 16SrRNA PCR was performed as reported earlier.[17]

Table 1: Primer sequences for Seven DNA repair genes which chosen for gene expression analysis

Serial number	Name of the gene	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
1	RecN/ML1360	Forward 5'-GACTGTACTGACCGGCGAAA-3' Reverse 5'-CAGCACGGTTAGCTCCTGAT-3'	60	116
2	DnaJ1/ML2494c	Forward 5'-CACCGTGACCATTCCGGTTA-3' Reverse 5'-AGGATACGGCCATCTGAGGT-3'	60	120
3	ML1105	Forward 5'-GGTTGGTGTCCGAGTACGTT-3' Reverse 5'-TACAACACCGTGGCTGAACC-3'	60	119
4	ML0603	Forward 5'-GCTGAACGCTGTTGGTTCTG-3' Reverse 5'-CTGTGATAACGCTGAACCGC-3'	60	108
5	ML0202	Forward 5'-CCTGCTGACGGGACTATGAC-3' Reverse 5'-GCCATCCTGAAAATCCGCAG-3'	60	120
6	RuvA/ML0482	Forward 5'-ATAGTGATGTCGCCTCGCTG-3' Reverse 5'-ACCTTGTCGCGTAACTCCAG-3'	60	85
7	RecA/ML0987	Forward 5'-AACCTCTCGCCCAATCTGTG-3' Reverse 5'-CCGAATGTTGCCCATTAGCG-3'	60	114

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Results

Genomic sequence annotations

A set of 96 DNA repair genes in the genome of *E. coli* and *M. tuberculosis* were considered as a reference and searched for their presence in *M. leprae* [Table 2, supplementary data]. This approach was adopted to identify the conserved nature of the DNA repair genes in Mycobacteriaceae and conversely, to identify the unique DNA repair genes in *M. leprae*. BLAST search on the protein database revealed the presence of 61 genes in the genome of *M. leprae* whose products detect orthologous DNA repair genes in *E. coli* and *M. tuberculosis*. Genbank annotations of the 61 genes identify 36 as characterized gene products (59%), 11 as hypothetical proteins (18%), and 14 as pseudogenes (23%). All these genes have orthologs in *M. tuberculosis* and 49 (80.32%) in *E. coli*. A set of 12 genes which are absent in *E. coli*, are present in *M. leprae* and Mycobacteriaceae. These include DNA ligases, DNA helicase II (*uvrD*), DNA helicase erCC3, Error-prone DNA polymerase DnaE2, DNA MMR protein *mutT,* and uracil DNA glycosylases. Functional annotation of all these proteins in DNA repair mechanisms is presented in Table 2.

Sequence comparison and phylogenetic analysis

A set of 11 hypothetical genes, namely *ML1105, ML1889, ML0202, ML0190, ML0603, ML2157, ML1351, ML1682, ML2698, ML1683,* and *ML1175* which are identified in the above approach were further searched for homologs across the prokaryotic databases using KEGG SSDB search with SW scoring matrix.[12] This was performed to identify the functional characteristics of the hypothetical proteins in relevance to DNA repair and to decipher the evolutionary relatedness with homologs in other bacteria. Multiple sequence alignment of these proteins with MUSCLE indicated that many of Mycobacteriaceae family members contain the conserved residues. All the close homologs that had high sequence identities are hypothetical proteins themselves and are identified as entities of Mycobacteriaceae family. These were selected to build a phylogenetic tree. The phylogenetic profiles were bootstrapped 100 times before constructing the trees. All the phylogenetic trees confirmed a close relationship between the 11 hypothetical proteins and proteins from the Mycobacteriaceae family. Hence, these hypothetical proteins are well conserved and might possess a functional role. Some of the closely related species matches include *M. haemophilum, M. tuberculosis, M. marinum,* and *M. kansasii*.

Annotation of ribosome binding sites and promoters

To identify the expression characteristics of the 11 hypothetical protein coding genes mentioned in sections above, presence of RBS and promoter like sequences in the 5' UTR were determined by multiple sequence alignment with promoter-like regions of other Mycobacterial homologs. A representative set of alignments for two hypothetical proteins with their transcription initiation sites, Shine – Dalgarno (SD) sequence and translational start points were aligned to their homologs in Mycobacteria [Figure 1]. Some of the hypothetical proteins demonstrate low similarities with their Mycobacterial counterparts. Although Mycobacterial promoters, for the most part, comprise of some indistinguishable segments from established bacterial promoters and occur upstream of and/or lie between the coding areas of two adjoining gene fragments; some much diverse promoter sequences concurrently exist, which direct the sequence interpretation and transcription in *M. leprae*. To check whether these hypothetical protein coding genes express in *M. leprae*, despite lacking canonical promoter regions, a set of 3 hypothetical proteins that indicated low similarity with their homologs in other mycobacteria, were chosen and qPCR was performed to identify gene expression.

Gene expression profiles from the *Mycobacterium leprae* whole transcriptome microarray

Transcriptome data were analyzed for 61 genes identified from the sequence based homology searches above and it was noted that transcripts corresponding to all the 61 genes were detected from the transcriptome data. A set of 60 nt length probes tiling every 10 nt and complementary to the transcripts of each of the 61 DNA repair genes in *M. leprae* (with mean signal-to-noise ratio cut-off value of ≥2), were analyzed. The signal intensities of each of the transcript was normalized with that of *16SrRNA* whose median signal intensity was 8.051386. The fold-change in average gene expression levels was obtained by dividing the *16SrRNA* signal intensity value with that of the expressed DNA repair genes followed by logarithmic transformation. It was noted that *ML1335c* demonstrated highest signal intensity and it was annotated as a pseudogene in *M. leprae* having seven stop codons. These observations correlate with the earlier findings on higher expression of pseudogenes and their implications in *M. leprae*.[19] It was noted that *RecN* which is primarily involved in homologous recombination process was overexpressed in the current experimental sample. However, the other genes contributing to this pathway are moderately expressed. The least expressed gene is *RuvA*, which has a signal intensity that is nearly equal to that of *16SrRNA*. A heatmap indicating expression levels of all the 61 DNA repair genes is represented in Figure 2.

Determination of gene expressions of a representative set of seven DNA repair genes by quantitative polymerase chain reaction

The gene expression profiles of 3 hypothetical protein coding genes (*ML1105, ML0202,* and *ML0603*) and 4 regular DNA repair genes (*RecN, DNAJ1, RuvA,* and *RecA*) from untreated patients' sample were analyzed using qPCR. qPCR assays were based on target-specific primers and a master mix containing SYBR Green I fluorescent dye that intercalates with double-stranded DNA (dsDNA/cDNA) that was generated during each progressive cycle of the PCR and emits a fluorescence signal which is quantitatively measured to track the amplification of cDNA. There is a quantitative relationship between the amount of starting template and the PCR product at the exponential phase of the PCR.[8]

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Table 2: Comparison of DNA repair genes of *Mycobacterium leprae* with *Escherichia coli* and *Mycobacteriaceae* family with focus on *Mycobacterium tuberculosis*

Name of protein (*E. coli*)	GI accession	Uniprot Id	*Mycobacteriaceae*	*M. tuberculosis*	Gene name in *M. tuberculosis*	*M. leprae* (*TN* strain)	Gene name in *M. leprae*	NCBI gene ID	GI accession	*Function/alternative name*
Base excision repair										
Adenine DNA Glycosylase										
MutY	16130862	P17802	Present	Present	Rv3589	Present	ML1920	910168	NP_302294	Adenine DNA glycosylase
Uracil DNA Glycosylase										
udgB	Absent		Present	Present	Rv1259	Present	ML1105 hypothetical protein	910195	NP_301808	Family 5 UDG
Ung	148149	P12295	Present	Present	Rv2976c	Present	ML1675c	910041	NP_302149	UDG
Mug	1789449	P0A9H1	Present	Absent	Absent	Absent				
AP endonuclease										
Nfo/end	16130097	P0A6C1	Present	Present	Rv0670	Present	ML1889c hypothetical protein	910601	NP_302271	Endonuclease IV with intrinsic 3'-5' exonuclease activity
XthA	16129703	P09030	Present	Present	Rv0427	Present	ML1931 pseudogene	910101		Exodeoxyribonuclease III
Nucleotide excision repair										
Excinucleases										
UvrA	16131884	P0A698	Present	Present	Rv1638	Present	ML1392	910525	NP_301990	ATPase and DNA damage recognition protein of nucleotide excision repair excinuclease UvrABC
UvrB	67474768	P0A8F8	Present	Present	Rv1633	Present	ML0483	909232	NP_301423	UvrABC system protein B, excinuclease ABC subunit B
UvrC	189038049	A1AC65	Present	Present	Rv1420	Present	ML0481	909230	NP_301421	UvrABC system protein C, excinuclease ABC subunit C
Mfd	1787357	P30958	Present	Present	Rv1020	Present	ML0252	908750	NP_301309	Transcription-repair coupling factor
Helicases										
UvrD	148212	P03018	Present	Present	Rv0949 uvrD1	Present	ML0153	908505	NP_301239	DNA helicase II
UvrD2	Absent		Present	Present	Rv3198c	Present	ML0637	909420	NP_301526	DNA helicase II paralog
ercc3/xpb	Absent		Present	Present	Rv0816c	Present	ML2157 hypothetical protein	908199	NP_302420	Helicase (eukaryotic)
Mismatch repair										
MutH	730086, 42065	P06722	Absent	Absent		Absent				DNA mismatch repair protein mutH, methyl-directed mismatch repair protein
MutL	42067	P23367	Present	Absent		Absent				MutL protein
MutS	17017340	P23909	Present	Absent		Absent				MutS

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Table 2: Contd...

Name of protein (*E. coli*)	GI accession	Uniprot Id	*Mycobacteriaceae*	*M. tuberculosis*	Gene name in *M. tuberculosis*	*M. leprae* (*TN* strain)	Gene name in *M. leprae*	NCBI gene ID	GI accession	*Function/alternative name*
vsr	16129906	P09184	Absent	Absent		Absent				Very short patch repair
sbcB	16129952	P04995	Absent	Absent		Absent				
XseA	148275	P04994	Present	Present	Rv1108c	Present	ML1940	910082	NP_302308	Exonuclease VII large subunit
XseB	89107292	P0A8G9	Present	Present	Rv1107c	Present	ML1941	910018	NP_302309	Exonuclease VII small subunit
Homologous recombination										
RadA	16132206	P24554	Present	Present	Rv3585	Present	ML0318c pseudogene	910137		
RecA	37362719	P0A7G6	Present	Present	Rv2737c	Present	ML0987	910009	NP_301732	RecA
RecB	16130724	P08394	Present	Present	Rv0630c	Absent				Exonuclease V (RecBCD complex), beta subunit
RecC	16130726	P07648	Present	Present	Rv0631c	Absent				Exonuclease V (RecBCD complex), gamma chain
RecD	16130723	P04993	Present	Present	Rv0629c	Absent				Exonuclease V (RecBCD complex), alpha chain
RecE	147536	P15032	Absent	Absent		Absent				RecE
RecF	147539	P0A7H0	Present	Present	Rv0003	Present	ML0003	910262	NP_301131	RecF
RecG	42669	P24230	Present	Present	Rv2973c	Present	ML1671c	910037	NP_302148	RecG
RecJ	887842	P21893	Present	Absent		Absent				Single-stranded DNA-specific exonuclease
RecN	42693	P05824	Present	Present	Rv1696	Present	ML1360	910489	NP_301970	Unnamed protein product
RecO	499369	P0A7H3	Present	Present	Rv2362c	Present	ML0633	909415	NP_301524	RecO
RecQ	147559	P15043	Present	Absent		Absent				RecQ
RecR	16128456	P0A7H6	Present	Present	Rv3715c	Present	ML2329c	908695	NP_302515	Gap repair protein
RecT	397681	P33228	Present	Absent		Absent				Unknown
RecX	16130605	P33596	Present	Present	Rv2736c	Present	ML0988	910283	NP_301733	Regulatory protein for RecA
RuvA	581226	P0A809	Present	Present	Rv2593c	Present	ML0482	909231	NP_301422	Unnamed protein product
RuvB	42903	P0A812	Present	Present	Rv2592c	Present	ML0483	909232	NP_301423	Unnamed protein product
RuvC	42175	P0A814	Present	Present	Rv2594c	Present	ML0481	909230	NP_301421	RuvC
Nonhomologous end joining										
Ku	Absent		Present	Present	Rv0937c	Present	ML2092 pseudogene	908303		
LigB	Present		Present	Present	Rv3062	Present	ML1747 pseudogene	910746		
LigC	Absent		Present	Present	Rv3731	Absent	Absent			
LigD	Absent		Present	Present	Rv0938	Present	ML2090 pseudogene	908652		
Translesion synthesis										
UmuC	84060801	P04152	Present	Absent		Absent				UmuC

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Name of protein (*E. coli*)	GI accession	Uniprot Id	*Mycobacteriaceae*	*M. tuberculosis*	Gene name in *M. tuberculosis*	*M. leprae* (*TN* strain)	Gene name in *M. leprae*	NCBI gene ID	GI accession	*Function/alternative name*
UmuD	85376582	P0AG11	Present	Absent		Absent				UmuD
DinB	16128217	Q47155	Present	Present	Rv1537 DinX	Present	ML1197 pseudogene	910299		DNA polymerase IV
DinP	Absent		Present	Present	Rv3056	Present	ML1739 pseudogene	910754		
Direct repair										
Ogt	84027827, 16129296	P0AFH0	Present	Present	Rv1316c	Present	ML1151c	910248	NP_301845	6-O-methylguanine-DNA methyltransferase, O-6-methylguanine-DNA-alkyltransferase. Methylated-DNA-protein-cysteine methyltransferase
AlkB	405945	P05050	Present	Present	Rv1000c	Present	ML0190 hypothetical protein	908584	NP_301263	AlkB
AlkA	112786	P04395	Present	Present	Rv1317c	Present	ML1152c pseudogene	910249		DNA-3-methyladenine glycosylase 2, DNA-3-methyladenine glycosylase II, 3-methyladenine-DNA glycosylase II, inducible, DNA-3-methyladenine glycosidase II
Ada	461468	P06134	Present (abseccus)	Absent		Absent				Regulatory protein ada, regulatory protein of adaptive response, contains: Methylated-DNA-protein-cysteine methyltransferase, O-6-methylguanine-DNA alkyltransferase
Phr	6006450	P00914	Present	Absent		Absent				CPD photolyase, DNA photolyase
Nucleotide pool										
MutT	16128092	P08337	Present	Present	Rv2985	Present	ML1682 hypothetical protein	910049	NP_302156	8-oxo-dGTP diphosphatase
MutT2	Absent		Present	Present	Rv1160	Present	ML1503 pseudogene	909535		
MutT3	Absent		Present	Present	Rv0413	Present	ML0301 pseudogene	908862		
MutT4	Absent		Present	Present	Rv3908	Present	ML2698 hypothetical protein	908257	NP_302721	Possible mutT4, mutator protein
RdgB	16130855	P52061	Present	Present	Rv1341	Present	ML1175 hypothetical protein	910273	NP_301857	dITP/XTP pyrophosphatase
MazG	16130688	P0AEY3	Present	Present	Rv1021	Present	ML0253 pseudogene	908752		
Dut	16131511	P06968	Present	Present	Rv2697c	Present	ML1028	910096	NP_301761	Deoxyuridine 5'-triphosphate nucleotidohydrolase

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Regulatory										
LigA	91211748	P15042	Present	Present	Rv3014c	Present	ML1705c	910789	NP_302174	NAD-dependent DNA ligase
LexA	67467382	P0A7C3	Present	Present	Rv2720	Present	ML1003c	910052	NP_301742	LexA repressor
PolI	147312, 42461	P00582	Present	Present	Rv1629	Present	ML1381c	910507	NP_301982	DNA polymerase I
Ssb	16131885	P0AGE0	Present	Present	Rv0054	Present	ML2684c	908269	NP_302712	Single-stranded DNA-binding protein
Other potential DNA repair genes										
AidB	12644215	P33224	Present	Absent		Absent				Protein AidB
Dam	16131265	P0AEE8	Present	Absent		Absent				DNA adenine methylase
DinF	89110765	P28303	Present	Present	Rv2836c	Absent				DNA-damage-inducible SOS response protein
DinF like	Absent		Absent	Present	Rv2090	Present	ML1335 pseudogene	910459		
DinG	89107650	P27296	Present	Present	Rv1329c	Absent				ATP-dependent DNA helicase
DinI	89107907	P0ABR1	Absent	Absent		Absent				DNA damage-inducible protein I
DinJ	89107101	Q47150	Present	Absent		Absent				predicted antitoxin of YafQ-DinJ toxin-antitoxin system
DnaE	146663	P10443	Present	Present	Rv1547	Present	ML1207	910310	NP_301875	DNA polymerase III holoenzyme, alpha subunit
DnaE2	Absent		Present	Present	Rv3370	Present	ML0416 pseudogene	909155		
DnaJ	16128009	P08622	Present	Present	Rv0352	Present	ML2494	908467	NP_302611	Chaperone protein DnaJ
DnaN	145761	P0A988	Present	Present	Rv0002	Present	ML0002	908144	NP_301130	DNA polymerase III beta-subunit
DnaQ	147679	P03007	Present	Present	Rv3711c	Present	ML2325c pseudogene	908707		DNA polymerase III epsilon subunit
DnaT	16132183	P0A8J2	Absent	Absent		Absent				DNA biosynthesis protein (primosomal protein I)
DnaX	118808	P06710	Present	Present	Rv3721c	Present	ML2335c	908684	NP_302521	DNA polymerase III subunit tau; contains: DNA polymerase III subunit gamma
Fpg (MutM)	146545	P05523	Present	Present	Rv2924c	Present	ML1658c	910013	NP_302139	Formamidopyrimidine-DNA glycosylase
HelD	146328	P15038	Present	Absent		Absent	Absent			Helicase IV
HolA	145729	P28630	Present	Present	Rv2413	Present	ML0603 hypothetical protein	909387	NP_301508	DNA polymerase III delta subunit
HolB	145799	P28631	Present	Present	Rv3644c	Present	ML0202 hypothetical protein	908611	NP_301270	DNA polymerase III delta prime subunit
HolC	145537	P28905	Absent	Absent		Absent				DNA polymerase III chi subunit
HolD	147387	P28632	Absent	Absent		Absent				DNA polymerase III psi subunit
HolE	145787	P0ABS8	Absent	Absent		Absent				DNA polymerase III theta subunit

Contd...

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Table 2: Contd...

Name of protein (*E. coli*)	GI accession	Uniprot Id	*Mycobacteriaceae*	*M. tuberculosis*	Gene name in *M. tuberculosis*	*M. leprae* (*TN* strain)	Gene name in *M. leprae*	NCBI gene ID	GI accession	*Function/alternative name*
HupA	16131830	P0ACF0	Present	Present	Rv2968c	Present	ML1683 hypothetical protein	910050	NP_302157	HU subunit alpha
HupB	16128425	P0ACF4	Absent	Absent	Absent	Absent	Absent			HU subunit beta
Mpg	Absent		Present	Present	Rv1688	Present	ML1351 hypothetical protein	910482	NP_301965	3-methyladenine DNA glycosylase
Nei	16128689	P50465	Present	Present	Rv3297	Absent				5-formyluracil/5-hydroxymethyl UDG
Nei2	Absent		Present	Present	Rv2464c, Rv0944	Present	ML1483 pseudogene	909513		
Nfi	90111673	P68739	Absent	Absent	Absent	Absent	Absent			Endonuclease V
Nth	16129591	P0AB83	Present	Present	Rv3674c	Present	ML2301c	908145	NP_302496	DNA glycosylase and AP lyase (endonuclease III)
PolII	147318	P21189	Absent	Absent		Absent				DNA polymerase II

M. leprae: *Mycobacterium leprae*, *M. tuberculosis*: *Mycobacterium tuberculosis*, UDG: Uracil DNA glycosylase, diTP: Deoxyinosine triphosphate, XTP: Xanthosine triphosphate, AP: Apyrimidinic, *E. coli*: *Escherichia coli*, GI: Gastrointestinal

Standard curves to determine the amplification efficiency of the selected genes in quantitative polymerase chain reaction

Before testing on clinical samples, pure stocks of bacterial reference DNA of *M. leprae* (Br4923 strain) was used to construct standard graphs. These graphs were developed to validate the assays, identify lower detection limit and determine error rates in the qPCR experiments. Standard curves were constructed by estimating threshold cycle values for seven 10-fold serial dilutions of purified *M. leprae* DNA ranging from 0.5 ng to 7.8 pg for each qPCR assay [Table 3 and Figure 3]. Optimal fluorescence thresholds were chosen based on the common practice that it should be positioned on the lower half of the fluorescence accumulation curves plot from the 10-fold dilutions and was used both to calculate the Ct for standard curve fitting and Ct for all the 10 clinical samples in the study.

Relative abundance of DNA repair gene transcripts in M. leprae RNA from clinical isolates

qPCR of *16SrRNA* served as a positive control, imparting incremental sensitivity over assays based on the detection of a single or multiple copies of genomic sequences, since each cell contains 1000–10,000 copies of rRNA. Real time PCR was performed in duplicates for each of the 10 skin biopsies. The mRNA expression levels of all the 10 genes in clinical isolates from newly diagnosed untreated leprosy cases reveal a range of threshold fluorescence values. The average Ct values for all the 10 samples for each of the gene was represented in Figure 4.

Comparative analysis of expression levels of all the seven genes using qPCR and microarray data suggested that *RecA*, ML0202 and ML0603 indicated substantial correlation. Rest of the genes in the analysis revealed a poor correlation with observations from microarray data [Figure 5]. *RuvA* indicated increased expression in qPCR and low intensities in microarray data. One of the possible reasons for this observation could be due to the selection of leprosy cases which are all highly bacillated providing high quantities of bacterial RNA. *RecA*, ML0202 and ML0603 indicated similar expressions in both qPCR and microarray data which suggests that ML0202 and ML0603 may have a significant functional role in the DNA repair pathways. The mean Ct values of each of the genes along with the normalized (delta Ct) values are represented in Table 4 and the microarray fold changes for the same set of genes has been represented in Table 5.

Discussion

The relevance of this comparative analysis is to provide the basis for investigating the putative genes and pathways detected in the genome of *M. leprae*. The presence and absence of DNA repair genes are discussed and predictions are made considering the particular aspects of the *M. leprae* among other known DNA repair pathways. Sequence annotations of DNA repair genes in *M. leprae* with insights from their orthologs in *E. coli* and *M. tuberculosis* enabled identification of potential DNA repair pathways. DNA repair genes were stratified based on their function in the following mechanisms: base excision repair, nucleotide excision repair (NER), MMR, recombination

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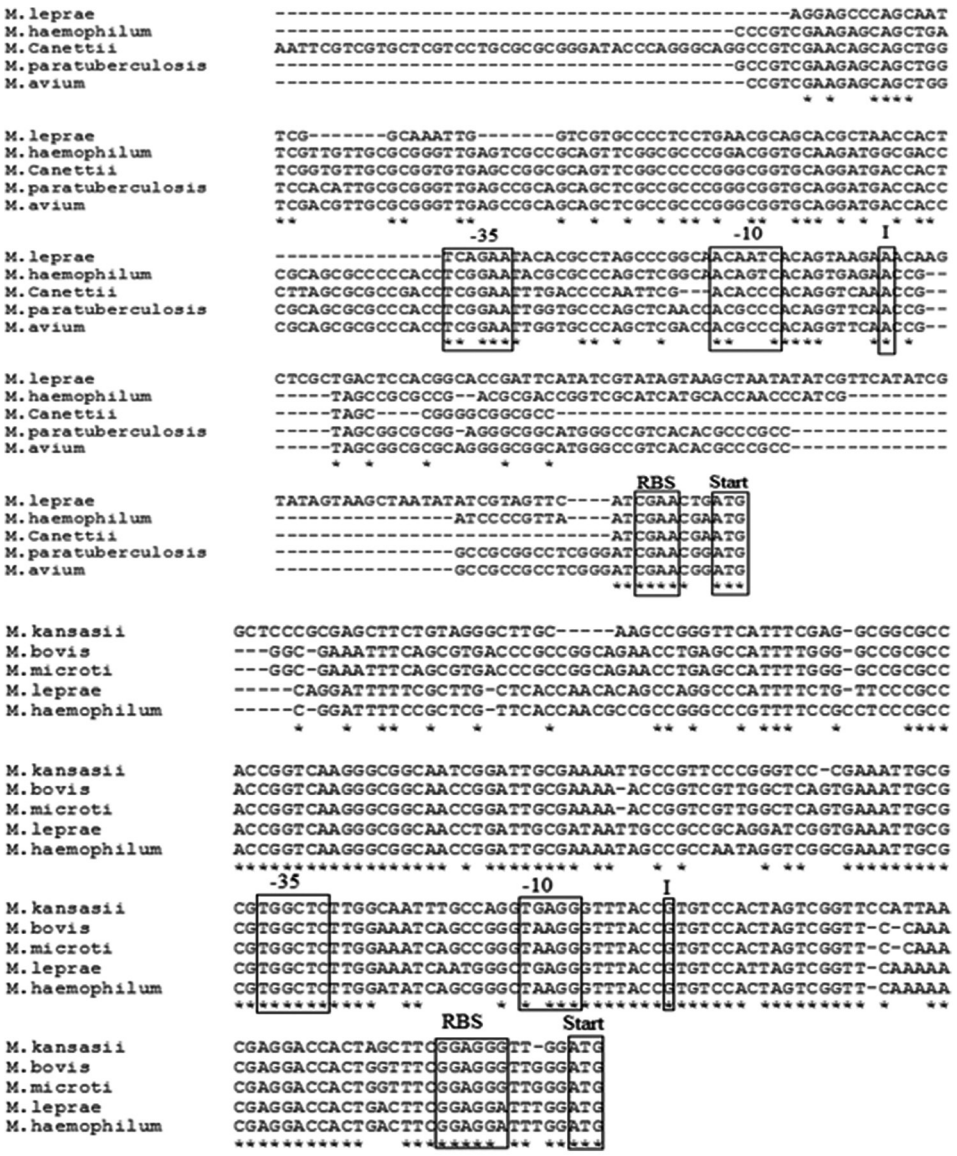

Figure 1: Promoter-like sequences upstream of transcribed *Mycobacterium leprae* hypothetical proteins ML1683 and ML0190: It shows representative alignments of promoter-like sequences for *Mycobacterium leprae* genes and their mycobacterial homologs which are within 200 nt upstream of the translational start point. Panel A and B represent the ML0190 and ML1683 upstream promoter-like regions containing -35 and -10 regions and initiation site (i) in relationship to their ribosomal binding sites and translational start codons (Start), respectively

repair, NHEJ, translesion synthesis (TLS), direct reversal, nucleotide pool, regulatory and other related processes.

Base excision repair

One of the primary mechanisms for the repair of alkylated bases is BER, which is initiated by one of the 3-methyladenine DNA glycosylases, *tagA* or *alkA*. A homolog of the *tagA* gene is present in *M. leprae* which includes 10 stop codons, splitting the corresponding locus into many reading frames and has been annotated as a pseudogene-(*ML0190*). A gene encoding "3-methyladenine DNA glycosylase" is also present in Mycobacteria and possess conserved regions throughout the Mycobacterial species. In *M. leprae*, it has been annotated as a hypothetical protein *ML1351*. Although no functional studies have been reported, the conservation of this gene across various species suggests its indispensable role. One of the most common and stable oxidation products in DNA is 8-oxo- 7, 8-dihydroguanine (8-oxo-G),[20] having a propensity to mispairing with adenine. Both modified bases act as substrates for the formamidopyrimidine-DNA glycosylase, known as *fpg* or *mutM*.[21] The *fpg* gene has been shown to be involved in the repair of DNA lesions induced by hydrogen peroxide in *E. coli*.[22] *M. tuberculosis* (H 37Rv) has four genes of the *fpg/nei* family of DNA glycosylases: *Rv2924c* annotated as *fpg* (*ML1658* in *M. leprae*), *Rv3297* annotated as *nei*, *Rv0944* (*ML0148* in *M. leprae*) annotated as a possible *fpg*, and *Rv2464c* (*ML1483* in *M. leprae*) annotated as a possible DNA glycosylase. Homologs of all four of these

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genes are found in the other Mycobacterial genomes and in *M. leprae* the loci corresponding to *Rv0944* and *Rv2464c* contain pseudogenes, whereas there is no equivalent of *Rv3297*. Endonuclease III (*Nth*) excises oxidative pyrimidines. A homolog of *Nth* is present in both the mycobacterial genomes and it is named as *ML2301c* in *M. leprae*.

Adenine can be incorporated rather than the cognate cytosine opposite 8-oxo-G during DNA replication, leading to G.C and T.A transversions. To contract this, the adenine DNA glycosylase (*mutY*) excises the mismatched pair, which also includes nucleotides on the complementary strand. The *mutY* gene in *M. leprae* is *ML1920* which has homologs that are identified in other Mycobacterial genomes as noted in earlier studies.[23] Uracil can also be found in DNA either because of misincorporation or deamination of cytosine. The archetypal family-1 Uracil DNA glycosylases/(ung) are specific to uracil in DNA and excise it from both double-stranded (ds) and single-stranded (ss) substrates.[24] The homologs of *udgB* from *E. coli* and *M. tuberculosis* are present in *M. leprae* as *ung* and ML1105. The second step in BER is the cleavage of sugar-phosphate backbone by an apurinic/apyrimidinic endonuclease. In *E. coli*, endonuclease IV (*Nfo*) and exonuclease III (*XthA*) produce a single-strand (ss) break at abasic sites by attacking the phosphodiester bond 5' to the site of base loss, leaving 3'OH groups. Homologs of *Nfo* have been identified in many Mycobacterial species and in *M. leprae*, it is annotated as hypothetical protein (*ML1889*). Similarly, *XthA* is also present in all Mycobacterial species except *M. leprae* where a corresponding pseudogene (*ML1931*) is found.

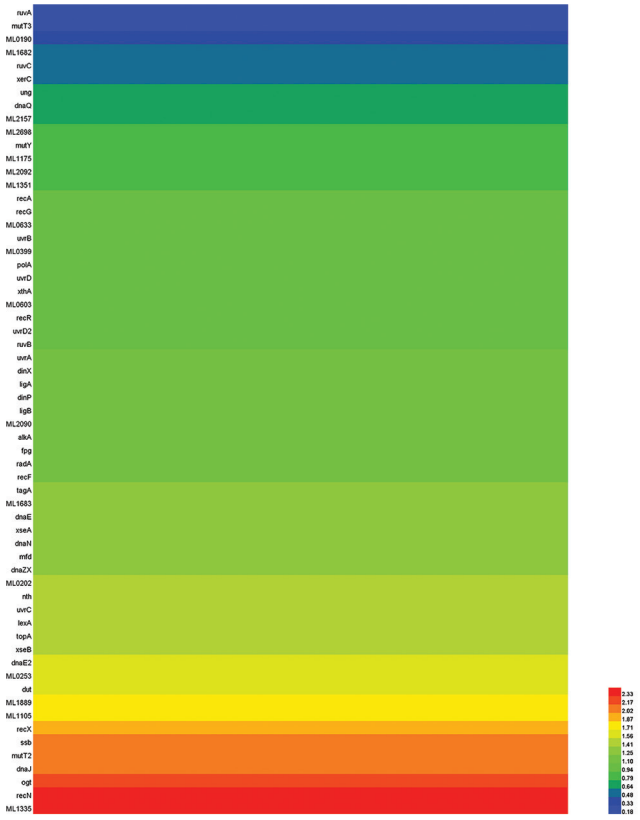

Figure 2: Heat-map of significant expression level changes in genes associated with DNA-repair

Nucleotide excision repair

This system recognizes the distortion in the double helix caused by lesions which can recognize a larger variety of base modifications. Removal of lesions from the intact oligonucleotide forms is facilitated by the sequential action of nucleases and helicases, followed by DNA polymerization and ligation by DNA ligase.[25] It includes proteins *uvrA*, *uvrB*, the nuclease *uvrC*, the helicase *uvrD* and the dsDNA translocase *Mfd*. Homologs of *uvrA*, *uvrB* and *uvrC* are present in all the Mycobacterial genomes including *M. leprae*, suggesting that this pathway of DNA repair is important to Mycobacteria. Despite the canonical *uvr* genes, an additional protein involved in the incision step of NER has been identified in *E. coli*, termed *cho* having sequence similarity with the N-terminal portion of *uvrC* and containing the domain for

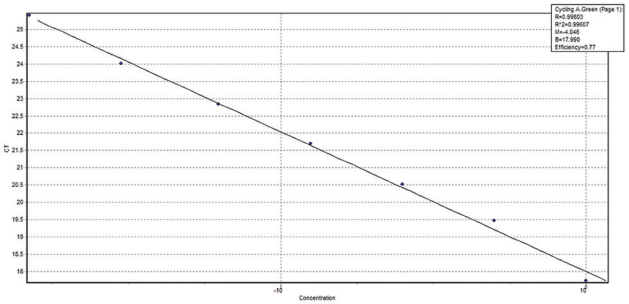

Figure 3: Standard graph of 16srRNA gene of *Mycobacterium leprae*

Table 3: Standard curves parameters and results for quantitative polymerase chain reaction assays of Mycobacterium leprae DNA

Concentration (ng/reaction)	RecN	Ogt	DnaJ1	RuvA	RecA	ML1105	ML1889	ML0202	ML0190	ML0603
0.5000000	14.45	16.44	13.75	18.34	13.14	15.13	15.90	13.86	18.14	16.56
0.2500000	14.80	16.23	14.06	18.53	13.17	14.74	15.66	13.96	17.50	16.71
0.1250000	15.34	17.51	15.11	19.72	14.01	15.88	16.84	14.87	19.37	17.84
0.0625000	16.53	18.11	16.05	20.75	15.53	17.38	17.78	15.92	19.83	18.67
0.0312500	17.74	19.97	16.76	21.93	16.21	18.08	18.47	16.79	21.00	19.88
0.0156250	18.48	20.80	17.91	23.05	17.28	19.51	19.87	18.11	22.38	21.21
0.0078125	20.39	21.72	18.90	23.80	18.11	20.75	21.58	19.45	-	21.62

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the 3' incision. The sequence of this protein is conserved throughout the Mycobacterial species, except *M. leprae*, where the corresponding locus is a pseudogene (ML0884c). Transcription-coupled repair is a sub-pathway of NER that selectively removes lesions from the transcribed strands, mediated by the transcription-repair coupling factor (*mfd*). Homologs of *mfd* have been identified in *M. leprae (ML0252)*; however, the actual function is yet to be deciphered. In *M. leprae*, there are two homologs of *uvrD*, annotated as *uvrD1* and *uvrD2*. While their role is not experimentally determined, their orthologs in *M. tuberculosis* interact with Ku, a component of the NHEJ pathway of DNA repair, stimulating the helicase activity. Thus, it may be that *uvrD1* is involved in multiple DNA repair pathways in Mycobacteria. While most of the Mycobacterial genomes have homologs for superfamily II helicases known in eukaryotes, the *M. leprae* gene *ML2157* encodes for ERCC3, a 3'-5' helicase and is reported as the first example of this gene in prokaryotes.[26]

Table 4: Summary of the qPCR results for selected DNA repair genes

Gene name	Mean Ct values	Delta Ct (Ct of target gene - Ct of reference gene)
16srRNA (reference gene)	25.95	-
recN	23.05	−2.9
dnaJ1	19.68	−6.27
ruvA	26.07	0.12
recA	25.50	−0.45
ML1105	22.41	−3.54
ML0202	25.92	−0.03
ML0603	24.83	−1.12

Ct: Cycle threshold

Table 5: Summary of the gene expressions from microarrays

Gene name	Mean expression values	Fold difference (gene/16srRNA)	Log2 values
16srRNA (reference gene)	8.051386		
recN	41.5385775	5.159183462	2.367143
dnaJ1	35.746767	4.439827751	2.150504
ruvA	9.104483	1.130796983	0.1773399
recA	15.478265	1.922434845	0.9429347
ML1105	28.226898	3.505843342	1.809762
ML0202	21.6997795	2.695160746	1.430371
ML0603	16.3059385	2.02523373	1.018088

Mismatch repair

The *mutS*/*mutL* complex recognizes DNA replicative errors or misalignments and will perform an excision of the section containing the mismatch.[27] *M. leprae* lacks a system for MMR, as *mutS*, *mutL* or *mutH* could not be identified and not even their homologs. The exonucleases recJ or exol (encoded by sbcB or xonA) are also absent in *M.leprae*. This indicates that Mycobacteria may possess alternative control over homologous recombination, possibly involving a *recA*-mediated strand transfer. *E. coli* and related enteric bacteria also possess a system known as very short patch repair that targets mismatched T.G base pairs arising from deamination of 5-methylcytosine, especially within motifs recognized by DNA cytosine methyltransferase. Repair is initiated by the Vsr protein which nicks the DNA immediately upstream of the mismatch pair, followed by synthesis of a short stretch (<10 nucleotides) of DNA by DNA polymerase I and ligation.[28] Both these genes are absent in *M. leprae*.

Homologous recombination

Recombination repair maintains genome integrity. In *E. coli*, two pathways, the *RecBCD* and *RecFOR* recruit RecA to single stranded DNA and provoke the repair of double stranded breaks or repair post replication daughter strand gaps respectively breaks or of postreplication daughter strand gap, respectively.[29] RecA plays a central role in recombination repair and homologous recombination by promoting homologous pairing and DNA strand exchange using ATP, involving the formation of a nucleoprotein filament.[30] In some Mycobacteria like *M. tuberculosis, recA* is encoded by an elongated gene containing an intein which is made active by protein splicing[31-33] and similar observations were noted in *M. leprae*. *M. leprae-recA* intein binds to cognate DNA and

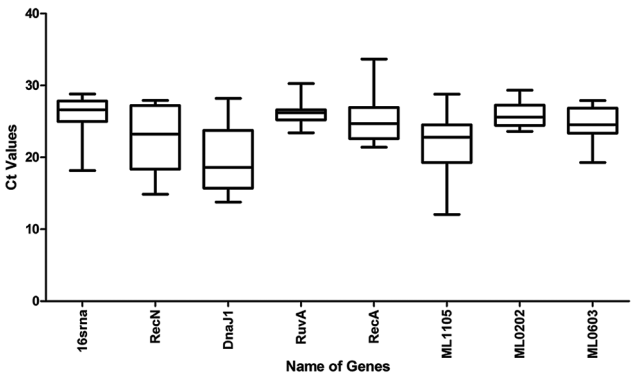

Figure 4: Mean Ct values of 4 DNA repair genes and 3 hypothetical protein coding genes along with *16SrRNA*

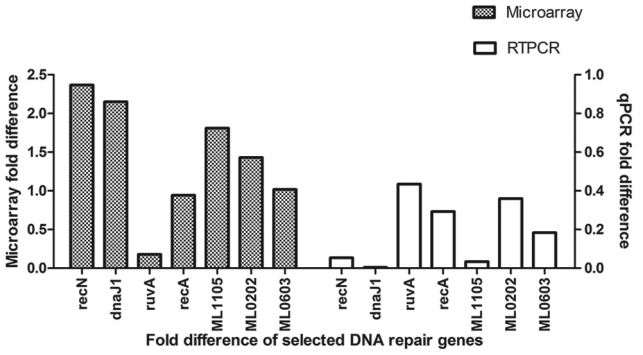

Figure 5: Comparison of gene expression fold difference between qPCR and microarrays. Genes are indicated by name whereas hypothetical proteins are indicated by their *M. leprae* accession numbers

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displays endonuclease activity in the presence of alternative divalent cations like Mg2+ or Mn2+.[34] In *E. coli*, several pathways exist for the initial processing of dsDNA breaks to single stranded substrates for recombination, each featuring the action of exonucleases and helicases. *M. leprae* possesses neither of these systems, but it does possess homologs of an archaeal exonuclease (*ML1155*) and helicase (*ML1312*) belonging to the *recB* family of exonucleases/helicases[34] in addition to *ML2157* and exonucleases (*sbcD* [*ML1119*], *xseAB*) which can perform the break-processing function. RuvABC and RecG complete the process of recombination by RecA. The RuvAB complex or the helicase RecG catalyze branch migration of Holliday junctions formed by the crossing over of strands from two DNA duplexes, and RuvC resolves this structure to allow separation of the DNA helices.[35] Homologs of each of RuvA, RuvB, RuvC, and RecG are present in *M. leprae*.

The functions of RecN and Rec X has not been elucidated to a substantial level in Mycobacteria and hence, their role in the repairing the double stranded breaks in *M. leprae* is unknown. *M. leprae* does not possess homologs of RecE and RecT genes. Homologs of RadA are present in many of the Mycobacterial species except in *M. ulcerans* and *M. leprae* consists of it in the form of a pseudogene (*ML0318c*).

Non-homologous end-joining

NHEJ also operates in some prokaryotes, including Mycobacteria,[36] but only Ku and ligase proteins are required.[8] Ku homologues are present in all the Mycobacterial species, with the single exception of *M. leprae* where it is present as a pseudogene (*ML2092*). Many Mycobacteria encode at least three different ATP-dependent ligases, known as LigB, LigC and LigD; expect in *M. leprae*, in which these genes are annotated as pseudogenes *ML1747* for LigB and *ML2090* for LigD. LigC is absent in *M. leprae*.

Translesion synthesis

In *M. leprae*, genes related to TLS are present as pseudogenes. DinB, DinP and dnaE2 coding genes are annotated as pseudogenes *ML1197*, *ML1739*, and *ML0416*, whereas other genes *umuC*, *umuD*, and *polB* are absent.

SOS Repair systems

The genes *umuC* and *umuD* form a complex UmuC/UmuD2, known as DNA polymerase V,[37] which is responsible for the induced mutagenesis through the SOS repair in *E. coli*. However, these polymerases are absent in *M. leprae*. The SOS inducible and error prone DNA polymerase IV (*dinB*) is involved in TLS in *E. coli*,[38] and thought to be doing the same regulatory function in *M. leprae*. The SOS induced mutagenesis in *M. leprae* has been proven to be promoted by enzymes encoded by operon including a second subunit of DnaE (the catalytic subunit of DNA polymerase III) called DNAE2.

The principal motivation for this study was to identify all the DNA repair genes present in the *M. leprae* genome, identify their expression from available microarray data and validate a representative set (especially the hypothetical proteins) using qPCR assay. Overall, 100% of the DNA repair genes were found to be transcribed as noted in microarrays. Different DNA repair pathways of *M. leprae* exhibited different levels of RNA expression. RNA expression was relatively higher for genes involved in the homologous recombination, whereas, the genes with a low level of expression were involved in the direct repair pathway. There were some differences in the levels of RNA expression detected by microarray and qPCR. The level of expression of hypothetical proteins involved in direct repair pathway detected by microarray were higher than the level from the same genes detected by qPCR, when compared to *16SrRNA* expression. This discrepancy might reflect the difference in the target length for both methods as well as the difference in the length of transcribed RNA.

The presence of promoter-like sequences in the 5'UTR of transcribed *M. leprae* hypothetical genes with translational start codons was investigated, using alignment of promoter like regions with that of Mycobacterial homologs. These promoters aligned very well with that of other Mycobacterial homologs and showed relationship to their-35 and-10 box, initiation site, RBS, and translational start codon. Although the results of this study indicate that some hypothetical proteins (supplementary data) having weak RBS sequences, some of the hypothetical genes like ML0190, ML1683 have intact ribosome-binding sequences of similar strength to the orthologs of Mycobacteriaceae. In addition, phylogenetic analysis also revealed that these hypothetical proteins from *M. leprae* are well conserved and might possess a functional role.

Functional annotation of most of the above-mentioned gene products using experimental approaches is vital to elucidate the DNA repair mechanisms in *M. leprae*. Understanding and targeting the DNA repair processes in *M. leprae* can be an important strategy for the development of potential future therapeutics for leprosy as they are essential for the survival at different stages of infections. During leprosy infection, different sets of genes play a vital role in maintaining the stability of the Mycobacterial genome; therefore, an improved understanding of the role of DNA repair in the pathogenesis of Mycobacteria may uncover the great possibility for the effective treatment against leprosy. Nonetheless, the majority of the *in silico* work should be confirmed experimentally, this work provides a profile of those genes responsible for the maintenance of genome stability, contributing to the understanding of the mechanisms of genome protection and mutagenesis in *M. leprae*. It also provides a useful framework for further investigations on the functions of these genes with the confirmation of their presence in microarray and qPCR experiments.

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Conflicts of interest

There are no conflicts of interest.

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