Molecular chaperons were also strongly expressed in *M. leprae*, which may be associated with the strong host immune response against these chaperones, such as HSP70, as previously reported [22,23]. Putative monooxygenase (ML0065), an orthologue of *M. tuberculosis* flavin monooxygenase *EthA*, which is reported to be an activator of thiocarbamide-containing antibiotics [24,25], was also strongly expressed. Since *katG*, another activator enzyme of thiocarbamide-containing antibiotics in *M. tuberculosis* [26], is a pseudogene in *M. leprae* [27], the role of *EthA* in *M. leprae* might be more important. Our BLASTP search revealed that ML2313, which showed the second strongest expression and contained no orthologue in the *M. tuberculosis* genome, was homologous to the transcription regulator *padR*. Specifically, *padR* is a negative regulator of phenolic acid decarboxylases *padA* and *padC*, both of which catalyze phenolic acid antibiotics to their vinyl derivatives [28]. Thus, the present analysis of *M. leprae* gene expression provides interesting possibilities of gene function and suggests a distinctive feature of *M. leprae* genes that might be related to the sensitivity to certain drugs. We have also tried to identify genes whose expression was as low as background. Although many hypothetical proteins were included in this group, further analysis will be necessary to elucidate suppressed genes by comparing gene expression of *M. leprae* infected in susceptible and non-susceptible hosts.

In prokaryotes, functionally related genes are transcribed in a single RNA strand forming an operon, and RNA expression from operons has been reported in *M. leprae* and *M. tuberculosis*. These operons include ribosome components [29,30] and genes involved in carbohydrate transport [31], which are essential for homeostasis, as well as genes related to virulence, such as entry operon (*mce*) [32], ESAT-6 and CFP-10 family secreted proteins [33], and heat shock proteins [5]. In the present study, we identified seven known operons and 11 novel putative operons, which included ribosomal proteins, ESAT-6 and CFP-10 family proteins, Snm proteins, and molecular chaperones. Ribosomal proteins are clustered in highly conserved operons which are preserved among prokaryotes including *M. leprae* [30] in order to function collectively as parts of a complex molecular machinery of the ribosome. ESAT-6 and CFP-10 family proteins are limited only to *Mycobacterium* species and play an important role in *Mycobacterium* virulence and host immune response [33–35]. Their distantly related protein, Snm, is thought as a component of the secretion system for ESAT-6 and CFP-10 family together with other proteins [36]. The multiple expressions of these mutually related gene groups as operons imply their involvement in the intracellular survival strategies and virulence of *M. leprae*.

Among the newly identified operon candidates, nucleotide reduction gene (*nrd*) operon (ML1734-ML1736) has been reported in other bacteria but never in *Mycobacterium* [17]. Further analysis of their promoter regions will determine the precise range of these putative operons.

In summary, we have analyzed *M. leprae* gene expression profile using DNA microarray and identified highly expressed genes and novel operon candidates. These data will serve as a basis for further investigation of the function of *M. leprae* genes in relation to its unique characteristics, such as long-term latency, inability to grow *in vitro*, and high proportion of pseudogenes.

4. Materials and methods

4.1. Bacterial strains and growth conditions

Hypertensive nude rats (SHR/NCrj-*rnu*), in which the Thai53 strain of *M. leprae* was actively grown [18,19], were kindly provided by Dr. Y. Yogi of the Leprosy Research Center, National Institute of Infectious Diseases, Japan. *M. leprae* was isolated as previously described [10]. Briefly, the footpads of the SHR/NCrj-*rnu* rats were homogenized in Hank's balanced salt solution (HBSS) containing 0.025% Tween 80. The sample was centrifuged at 700g and 4 °C for 10 min to remove tissue debris. The supernatant was treated with 0.5% trypsin at 37 °C for 1 h, followed by centrifugation at 5000g and 4 °C for 20 min. The pellet was resuspended in 10 ml of HBSS containing 0.025% Tween 80 and 0.25 N NaOH. Further incubation at 37 °C for 15 min was followed by centrifugation, then the pellet was resuspended in 2 ml of phosphate buffered saline (PBS). Two microliters of the solution was spread on a glass slide and subjected to acid fast staining to count the number of bacilli.

4.2. RNA extraction from M. leprae

M. leprae RNA was prepared as described [10,11]. Briefly, bacilli (2.8×10^{11} cells) were suspended in 2 ml of RNA Protect Bacteria Reagent (QIAGEN, Germantown, MD) and vortexed. After standing for 10 min at room temperature, the cells were collected and treated in another 2 ml of the same reagent; to this bacilli-containing mixture, 0.4 ml of 1.0 mm Zirconia Beads (BioSpec Products, Bartlesville, OK) and 0.6 ml of Lysis/Binding buffer, a component of the mirVana miRNA Isolation kit (Ambion, Austin, TX), were added. The mixture was homogenized at 3000 rpm for 3 min using a Micro Smash (TOMY, Tokyo Japan) followed by four freeze–thaw cycles. RNA was extracted according to the manufacturer's instructions (Ambion). The extracted RNA was treated with DNase I (TaKaRa, Kyoto Japan).

4.3. Array design and analysis

ORF array from NimbleGen Systems (Madison, WI) was used for array analysis. In this array, 20 different highly specific 60 mer probes designed for each of the 1605 ORFs were mounted with multiple control probes. Probes were designed based on the sequence and CDS information from Sanger Institute (http://www.sanger.ac.uk/Projects/M_leprae/). These probes were arranged into five blocks on a glass plate. As a result, a total of 160,500 probes were used to analyze RNA expression from *M. leprae* ORF. Twenty micrograms of total RNA from *M. leprae* was reverse-transcribed using SuperScript II (Invitrogen, Carlsbad, CA). The generated cDNA was incubated with 10 ng of RNase A (Novagen, Madison, WI) at 37 °C for 10 min, phenol-chloroform extracted, and precipitated with ethanol. For Cy3 labeling, 1 μg of ds-cDNA was incubated with 1 OD_{600} unit of Cy3-9mer Wobble primer (TriLink Biotechnologies, San Diego, CA) for 10 min at 98 °C. Then, 8 mmol dNTPs and 100 U of Klenow fragment (New England Biolabs, Ipswich, MA) were added, followed by incubation at 37 °C for 2 h. The reaction was stopped by adding 0.1 volume of 0.5 M EDTA, and the labeled cDNA was precipitated with isopropanol. Array analysis was performed as previously described [10].

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.micpath.2010.05.010.

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PLoS one

Detection of *Mycobacterium leprae* DNA from Archaeological Skeletal Remains in Japan Using Whole Genome Amplification and Polymerase Chain Reaction

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Abstract

Background: Identification of pathogen DNA from archaeological human remains is a powerful tool in demonstrating that the infectious disease existed in the past. However, it is very difficult to detect trace amounts of DNA remnants attached to the human skeleton, especially from those buried in a humid atmosphere with a relatively high environmental temperature such as in Asia.

Methodology/Principal Findings: Here we demonstrate *Mycobacterium leprae* DNA from archaeological skeletal remains in Japan by polymerase chain reaction, DNA sequencing and single nucleotide polymorphism (SNP) analysis. In addition, we have established a highly sensitive method of detecting DNA using a combination of whole genome amplification and polymerase chain reaction, or WGA-PCR, which provides superior sensitivity and specificity in detecting DNA from trace amounts of skeletal materials.

Conclusion/Significance: We have detected *M. leprae* DNA in archaeological skeletal remains for the first time in the Far East. Its SNP genotype corresponded to type 1; the first detected case worldwide of ancient *M. leprae* DNA. We also developed a highly sensitive method to detect ancient DNA by utilizing whole genome amplification.

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Introduction

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* (*M. leprae*) and has affected humans for millennia. Whole genome sequence analysis of *M. leprae* has revealed that the genome is 3.3 Mbp in size, with only 1,605 genes that encode proteins and 1,115 pseudogenes [1]. Trough comparative genome analysis of *M. leprae* strains from Brazil, India, Thailand and United States, remarkably little genomic diversity has been uncovered suggesting that leprosy has a single clonal origin [2,3]. Phylogeographic analysis of single nucleotide polymorphisms (SNPs) has revealed that *M. leprae* originated in Africa and spread to European and Asian countries and then worldwide along with human migrations and trade routes. Such geographic and temporal migration has also been demonstrated by SNPs analysis of ancient *M. leprae* DNA present in skeletal remains as old as 1,500 years ago [2,3].

Leprosy results in multiple deformities including progressive bone defects secondary to the peripheral neuropathy caused by *M. leprae* infection [4,5]. Additionally, in advanced cases *M. leprae* infection causes specific osteological deformations in the areas of the nasal aperture, anterior nasal spine and alveolar process on the premaxilla, cortical areas of the tibia and fibula, distal ends of the metatarsals and diaphyses of the phalanges that may include both direct and reactive changes [6,7,8,9,10]. Paleopathological diagnosis of leprosy has been made solely based on these macroscopic changes in skeletal remnants. However, it is occasionally difficult to make a definitive paleopathological diagnosis of leprosy solely based on osseous lesions, because syphilis, tuberculosis and other infectious and granulomatous diseases as well as carcinomas of nasal or oral origin will cause rhinomaxillary lesions similar to leprosy.

Direct detection of DNA remnants of pathogenic microorganisms from ancient human skeletons using polymerase chain reaction (PCR) is becoming a powerful molecular tool for clearly demonstrating the presence of pathogens within the skeletal remains [11]. This method provides an inestimable tool for investigating the temporal and geographical spread of infectious

diseases in human history, in addition to the anthropological interest in diagnosing infectious diseases in ancient remains. By the use of so-called palaeomicrobiology, it may be possible to elucidate the epidemiology of past infectious disease by reconstituting the distribution of infected individuals, which is especially important in the field of emergence and re-emergence of infectious diseases [11]. Moreover, it may also be possible to track the genetic evolution of the pathogenic microorganisms.

A study employing paleomicrobiology was first reported in 1993, demonstrating the presence of *Mycobacterium tuberculosis* (*M. tuberculosis*) DNA using PCR from an ancient human skeleton [12]. Detection of *M. leprae* DNA was reported the next year [13] from ancient bone dating from 600 AD, followed closely by several other reports [14,15,16,17,18,19,20,21]. However, all of these studies used European and Middle Eastern archaeological materials from countries such as England, Germany, Denmark, Poland, Hungary, Czech, Croatia, Turkey, Israel and Egypt, but not from the Far East Asian countries. This may be related to the superior preservation of ancient buried skeletons in these countries compared to that in more a humid atmosphere with a relatively high environmental temperature, such as is commonly found in Asia [22].

Recently, we have obtained permission to examine skeletal remains excavated in Japan with paleopathological evidence of leprosy In the present study, we demonstrate *M. leprae* DNA and SNPs from skeletal remains. We also describe a highly sensitive whole genome amplification (WGA)-PCR method that may be suitable for detecting trace amounts of ancient microbial DNA.

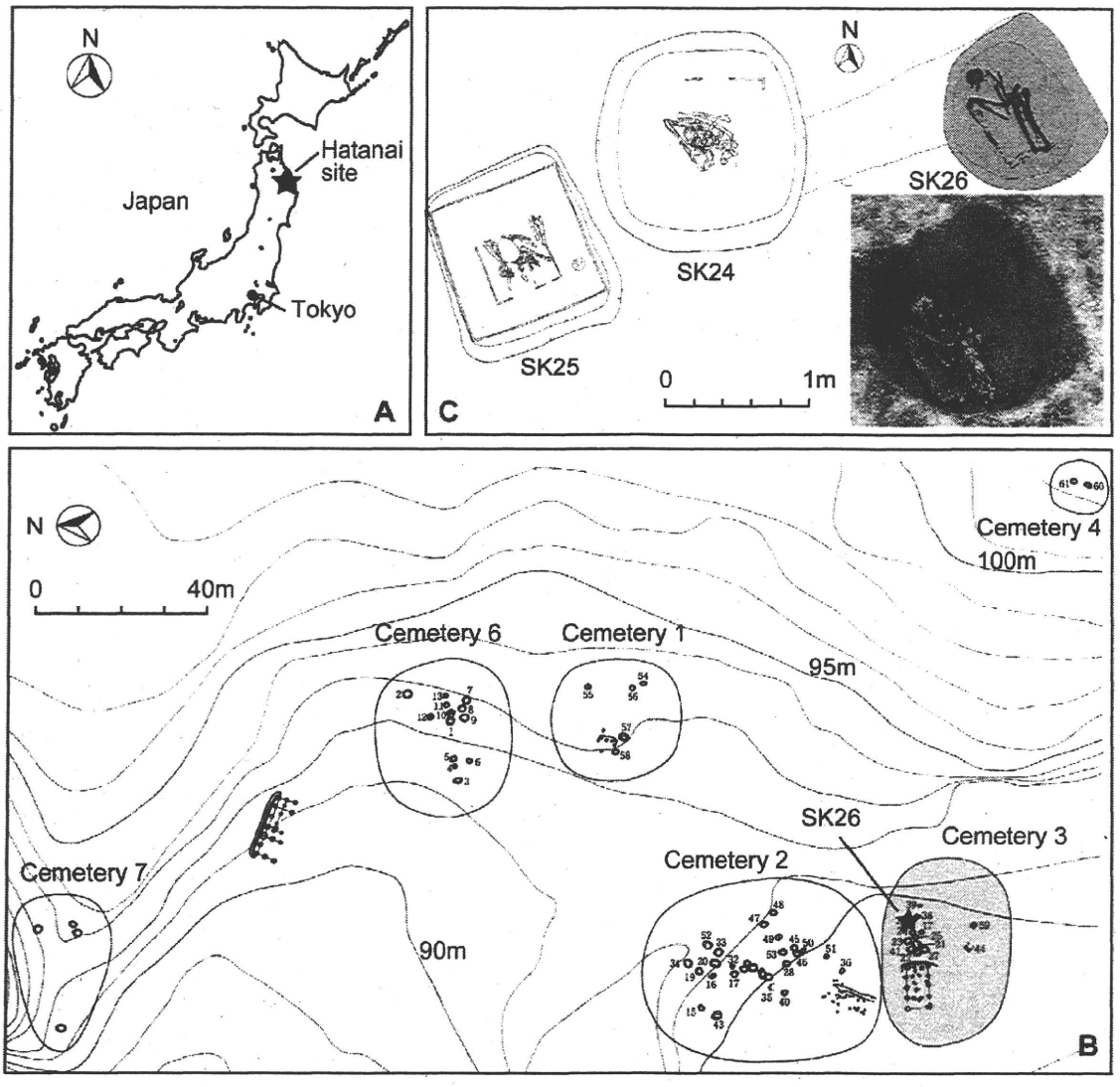

Figure 1. Geographical representation of excavation site. Location of *Hatanai* site in northeastern Japan (A). A map of seven cemeteries and the site where SK26 was excavated (B). A grave pit of SK26 at excavated (C) Cemetery 5 (not shown in this map) locates 50 m south of cemetery 2. All these figures were modified with permission from reference [23].
doi:10.1371/journal.pone.0012422.g001

Materials and Methods

Archaeological background and osteological description

The materials used in this study were archaeological skeletal remains excavated from the *Hatanai* site (N40°22′, E141°29′) in Aomori prefecture, in the northeastern part of Honshu Island in Japan (Fig. 1A). The *Hatanai* site consists of several strata including artifacts and remnants from the prehistoric Initial-Middle Jomon (ca. 8000-4500 BP) to pre-modern Edo period (1603–1867). All skeletal materials are stored in the Tohoku University Museum, Sendai, Japan.

The present material (Grave No. SK26) was buried at a gravel pit of an Edo-period cemetery in a farm village, in which was found about 50 human skeletal remains (Fig. 1B). This corpse was lying on its left side and hunching its back, with the elbow, hip and knee joints closely flexed inside the burial pit (Fig. 1C). It is not clear whether or not SK26 was placed in a wooden coffin. The SK26 skeleton had only one *Kouki-tsuhou (Kangxi-tongbao)*, a metallic currency used during the rule of the Emperor Kangxi (reign in 1661–1722) of the Qing dynasty (1616–1912) in China, as grave goods. Accordingly, the age of this burial is estimated as the middle 18th to the early 19th century, as supposed by evidences from other graves [23].

SK26's skull was almost perfectly intact except for part of the cranial base and parietal bones (Fig. 2). Although its main long bones were intact, many of their epiphyses and all the hand/foot bones were missing within the burial matrix of the postmortem environment. The sex of this skeleton was assessed as male on the basis of cranial morphologies, such as anterior decline of the frontal bone, projection of the superciliary ridge, inferior prominence of the mastoids, and developments of the superior nuchal line and the external occipital protuberance. But its bilateral pelvis was incompletely preserved and therefore unavailable for sex and age determination.

The age at death was estimated from dental attrition and fusions of the cranial sutures. Surviving permanent teeth were worn to dentine, including the 3rd molar. In the calvarium, the unions of all sutures in the internal table were finished; coronal and lambdoid sutures of the external table had incomplete fusing. These dental and skeletal evidences suggest that of the age of death, SK26 was middle-aged, in the range of 30–50 years old.

Palaeopathological observations and diagnosis

The facial cranium of SK26 showed several typical symptoms of leprosy, suggestive of lepromatous type. In the rhinomaxillary area, rounding deformation and abnormal enlargement of the nasal aperture, disappearance of the anterior nasal spine, and severe atrophy of the alveolar process were clearly observed (Fig. 2). The palatal process showed degenerative changes, and alveolar bones at the anterior teeth were closed by antemortem loss of all upper incisors and the right canine. The incisor foramen in the hard palate had also disappeared due to osseous resorption; for maxillary atrophy, the vertical thickness of the palatal bone was about nine millimeters on the right side of the alveolar process, and at the same portion on the left, only six millimeters at the maximum value.

These features of craniofacial malformation, especially the rhinomaxillary syndrome, are specific to lepromatous leprosy but essentially distinct from other facial-deforming diseases such as craniofacial tuberculosis and syphilis, a treponematoses [7,8]. Although the nasal, maxillary and palatal lesions in craniofacial tuberculosis closely resemble those in lepromatous leprosy, cranial involvements of tuberculosis are extremely rare in adult patients and mainly appear in young children, often with lesions of the mandible. In tertiary syphilis, rhinomaxillary deformations accompany several

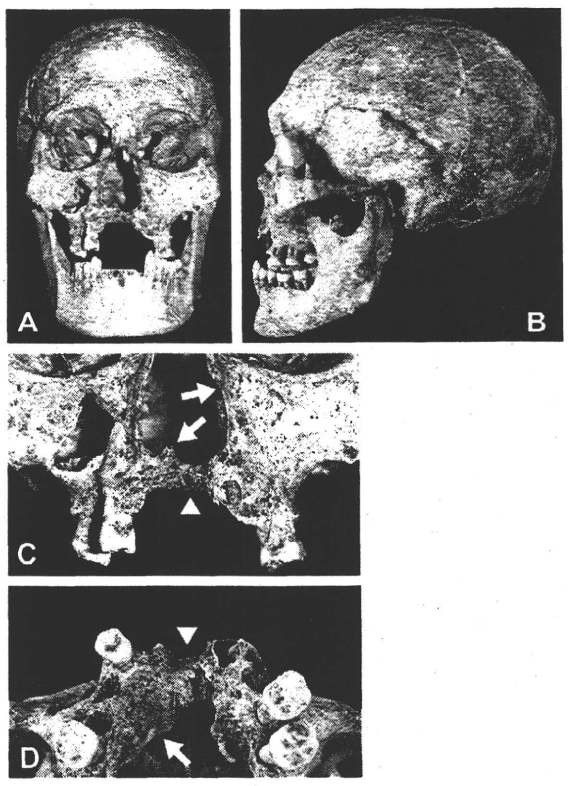

Figure 2. Macroscopic view of the skeletal lesion in the skull of sample SK26. Frontal view (A) and left side view (B) of the skull. Closer view of the nasal aperture and maxillary palate (C) and upward view of the palate (D). Arrows in panel C indicate erosive deformity of the nasal aperture and disappearance of the anterior nasal spine. Arrowheads in panels C and D indicate severe atrophy of alveolar bone in the maxilla/palatal process with loss of anterior teeth. An arrow in panel D denotes the locus of sample No. 1 shown in Table 1 and Figure 2.
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osteo-periostitic lesions and focal destructive remodeling (caries sicca) caused by the bone gummas in the cranial vault [7,8]. From differences in these pathological features, the facial deformities of SK26 were diagnosed as leprous symptoms.

In the bilateral tibiae and fibulae of this skeleton, slight periostitic changes were seen on the cortical surfaces of the diaphyses. The SK26 skeleton had lost its hand and foot bones from the long-term burial conditions, making it impossible to confirm any pencil-shaped recessions in the distal ends of the metacarpals, metatarsals or phalanges, which are typical leprous lesions.

Sampling of skeletal remains

Sampling was performed at the Tohoku University Museum, where no other leprous materials were stored. Skeletal samples were taken from the affected area as summarized in Table 1 using a small-sized rotating electric saw (Mini router, Kiso Power Tool, Osaka, Japan). Two samples were also taken from another skeleton (SK20; middle-aged male) found in the same cemetery, which had no leprous changes as a negative control for DNA purification and PCR analysis. One premolar root was extracted from another skeleton (SK16; middle-aged female) as a positive control for human DNA recovery. Sterile materials were used for the sampling to avoid possible contamination. Ethical

Table 1. Skeletal samples.

No.	Material reference	Sampling site	Paleopathological evidence	Sample weight (mg)
1	SK26	Maxillary palate, right	Erosion/atrophy	9.7
2	SK26	Inner surface of nasal cavity, right	Erosion/atrophy	6.0
3	SK26	Maxillary palate, left	Erosion/atrophy	4.6
4	SK26	Fibular diaphysis, right	Periostitis	22.1
5	SK20	Maxillary palate, right	N.R.	4.5
6	SK20	Inner surface of nasal cavity, right	N.R.	6.8
7	SK16	Lower 1st premolar root, right	N.R.	3.4

N.R.: No remarkable change.
doi:10.1371/journal.pone.0012422.t001

Table 2. PCR primer sequences.

Gene name	Synonym[a)]	Forward (5′-3′)	Reverse (5′-3′)	Length (bp)
Nested primer				
hsp-70, 1st	ML2496	gggctgtccaaggaagag	cgtcaaccacatcgtcagtag	391
(dnaK) 2nd		tcgtcaaggaacaacggg	cgtcaaccacatcgtcagtaga	261
16S rRNA, 1st	MLP000016	agagtttgatcctggctcag	tgcacacaggccacaaggga	1039
(rrs) 2nd		cggaaaggtctctaaaaaatctt	catcctgcaccgcaaaaagctt	171
SNP1, 1st	14,676	atttccaggtcttgtgcg	aggtcttcccaggacacc	351
2nd		aatggaatgctggtgagagc	caatgcatgctagccttaatga	194
SNP2, 1st	1,642,875	ttagtcaacatcgttagcagccc	actcataagcacggtgtctttgc	403
2nd		tgctagtttaaccgagtactgcta	gtagtagtcttccaagttgtggtg	189
SNP3, 1st	2,935,685	cgagcataatcgtaggcg	aaatgtggtcacctgggc	510
2nd		atctggtccgggtaggaatc	accggtgagcgcactaag	180
Gene				
cspA	ML0198	gaactgtgaagtggttcaacg	agcgaactccagtggcttg	186
hisE	ML1309	gaccttcgaggatctgttcg	atagacgtcatcgagcgaca	247
purM	ML2205	aaactcacgtccgtaccttc	agcacggtcagtatcttcag	233
cpsA	ML2247	ggcaccttcaccaacctaga	ccaacttaggatccgcttga	511
Pseudogene				
pseudo scoB	ML0434	tggaacacctcgtcgtatgtgg	tataagtggcaccgccgaactc	201
pseudo speE	ML0476	tcgcaacttcactgatcgtc	gtctggcaccaataccgagt	465
pseudo REP	ML0794	aaagacggagactacgatg	gtttagaaggttggtcgttg	191
pseudo ahpD	ML2043	tcaacatggcgatctgcattc	tgcgtgaccttacaacgct	200
Non-coding region				
	348,457	tggactcgatgttgaagtg	tgcttagctatgcagtgag	202
	1,593,211	catcgagtccaagctcaac	tgccgatgattacatcatcc	191
	2,134,972	cggaatcctgttgacgtgtt	cggcgctaacaactatcctc	242
	2,152,288	ccgatatgttcggtagtcgt	gcatcgatatcgccttcag	241
	2,307,322	ggttcaccggaagagttgg	cgcgacgactaagccagtag	242
	2,546,884	tcaatatggcttcctatgttgc	gctgcattaatccatgattcg	202
	2,551,060	acattcgagaccagctaccg	ttccgcttggaggataattg	194
	2,664,658	tgagcttgccgattacgatt	gccattgaactggccatc	227
	2,858,681	atgttggttgagcttggac	ttgcttagctatgcagtgag	217

Numbers in SNP nested primer indicate the coordinate location of the SNP. Numbers in non-coding regions indicate coordinate position of the primer within *M. leprae* genome (http://genolist.pasteur.fr/Leproma/).
doi:10.1371/journal.pone.0012422.t002

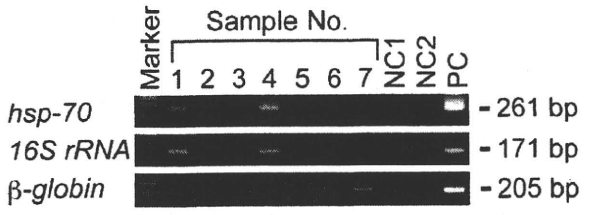

Figure 3. PCR detection of *M. leprae* DNA from skeletal samples. PCR analysis was performed using *M. leprae*-specific *hsp-70* and *16S-rRNA* primers for the DNA samples listed in Table 1. PCR products were evaluated by 2% agarose gel electrophoresis. Human *β-globin* gene was also PCR amplified as a control for DNA preparation from a premolar root. NC1: a negative control for DNA purification in which DNase/RNase-free water was used as a sample for DNA extraction; NC2: a negative control for PCR in which DNase/RNase-free water was used instead of a DNA sample for PCR reaction; and PC: positive control DNA from *Thai 53* strain of *M. leprae*.
doi:10.1371/journal.pone.0012422.g003

approval to work with the material was obtained from the review board at National Institute of Infectious Diseases, Japan. A permission to obtain the sample materials was granted by Tohoku University.

DNA preparations

DNA was purified using a QIAamp DNA Micro Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol with some modification. Briefly, 500 µl of Buffer ATL was added to the skeletal samples placed in a 2 ml test tube. The tube was then filled with 1.0 mm diameter Zicronia beads (Bio Space Products, Inc, South Lancaster, MA) and homogenized using Micro Smash MS-100 (TOMY, Tokyo, Japan) at 3,000 rpm for 5 min at 4°C [24,25]. Samples were then frozen at −80°C, thawed at 37°C and homogenized for five times. The supernatant was subsequently treated with 40 µl of Proteinase K (20 mg/ml) and incubated overnight at 56°C. The samples were frozen and thawed, vortexed in 400 µl of Buffer AL then 400 µl of ethanol was added and incubated for 5 min. Then the mixture was applied to a QIAamp MinElute column and centrifuged at 6,000× g for 1 min at room temperature. Flow-through was discarded and the column was subsequently washed and DNA was eluted by adding 20 µl of Buffer AE and centrifugation at 20,000× g for 1 min.

Polymerase chain reaction (PCR) and DNA sequencing

Touchdown PCR was performed using a PCR Thermal Cycler DICE (TaKaRa, Kyoto, Japan) as described previously [26]. PCR primers were designed to be specific to the *M. leprae* genome and were used in previous studies [24,27,28]; all the primers are listed in Table 2. The PCR products were analyzed by 2% agarose gel electrophoresis. Nested PCR was used to amplify *hsp-70*, *16S rRNA* and SNPs. Some of the results were confirmed in an independent laboratory.

The DNA sequence was analyzed using an ABI PRISM 310 Genetic Analyzer and GeneScan Collection software (Applied Biosystems).

Whole genome amplification (WGA)

A GenomePlex Whole Genome Amplification kit (Sigma, St Louis, MO) was used to amplify genomic DNA samples according to the manufacturer's protocol [29]. Briefly, 1 µl of 10× Fragmentation Buffer was mixed with 10 µl of DNA solution and incubated for 4 min at 95°C. After cool down, 2 µl of 1× Library Preparation Buffer and 1 µl of Library Stabilization Solution were added and incubated for 2 min at 95°C. Then 1 µl of 1× Library Preparation Enzyme was added and incubated at 16°C for 20 min, at 24°C for 20 min, at 37°C for 20 min and at 75°C for 5 min to ensure library preparation. To amplify genomic DNA, 7.5 µl of 10× Amplification Master Mix, 47.5 µl of Nuclease-free Water and 5 µl of Jumpstart *Taq* DNA polymerase were added and heated at 95°C for 3 min, then 14 cycles of two-temperature amplification was employed at 94°C for 15 sec and 65°C for 5 min using a PCR Thermal Cycler DICE (TaKaRa). The quality of the WGA products was evaluated by 1% agarose gel electrophoresis.

Others

All experiments were performed in a biosafety level 2 (BSL2) laboratory where samples were handled in a safety cabinet using disposable sterile materials to avoid any contamination.

Results

Demonstration of *M. leprae* DNA by PCR and DNA sequencing

The purified DNA from seven different sites of skeletons from three individuals was subjected to PCR amplification of the *M. leprae*-specific *hsp-70* gene and *16S rRNA* genes. A nested PCR protocol to amplify the *M. leprae*-specific *16S rRNA* region was developed in our laboratory. Among four samples taken from

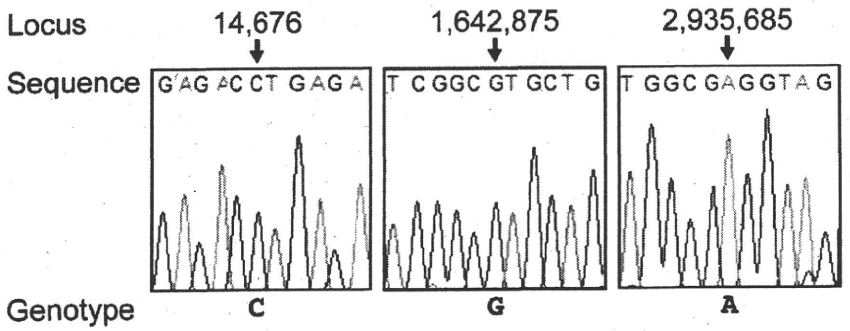

Figure 4. Sequence analysis of the three reported SNPs of *M. leprae* DNA. Each locus was PCR amplified and sequenced as described in the Materials and Methods.
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leprous lesions, a specific positive signal was derived from sample No. 1 (right maxillary palate of SK26) and No. 4 (right fibula of SK26), but not from the others (Fig. 3). Samples taken from control bones were all negative. Human *β-globin* DNA was detected by PCR only from sample No. 7 (lower premolar root of SK16).

The specificity of these PCR amplifications was confirmed by DNA sequencing of the PCR products purified from agarose gel. Basic Local Alignment Search Tool (BLAST) search of the DNA sequence was a 100% match with the reported *M. leprae* sequence for both *hsp-70* and *16S rRNA* as expected (data not shown). The results clearly confirmed that *M. leprae* was involved in the skeletal lesions of SK26 diagnosed as lepromatous leprosy.

SNP analysis of the *M. leprae* DNA

To determine the possible origin of the *M. leprae* found in this skeleton, we analyzed single-nucleotide polymorphisms (SNPs) at 3 reported loci in the *M. leprae* genome [2,3]. PCR amplification followed by direct sequencing identified the sequence at the 3 loci. Positions 14,676, 1,642,875 and 2,935,685, were "C", "G" and "A", respectively (Fig. 4). This genotype of *M. leprae* DNA is reported as SNP type 1, which is the dominant type in India and Southeast Asia [2,3].

Evaluation of WGA-PCR method

We have attempted to amplify other regions of the *M. leprae* genome using PCR primers we have designed previously [27] and found that the sensitivity to detect *M. leprae* DNA from ancient samples is significantly lower than from the clinical samples we analyze in our laboratory. To solve this problem, we have applied WGA to amplify whole genomic DNA purified from skeletal samples. Purified DNA from sample No. 1 was subjected to WGA and the DNA before and after WGA was compared by PCR using 19 different PCR primers targeting open reading frames, pseudogenes and non-coding regions of the *M. leprae* genome [24,27]. WGA-amplified DNA was positive for 15 sets of PCR primers, while *M. leprae* genomic DNA was detected by only 7 primer sets in the original DNA sample (Fig. 5), showing that WGA-PCR has superior sensitivity over conventional PCR.

Discussion

We have demonstrated *M. leprae* DNA in excavated human skeletal remnants from Japan by both PCR analysis and DNA sequencing. We also sequenced three SNPs in the *M. leprae* DNA and confirmed that the bacilli are of the dominant type in Southeast Asia. Although *M. leprae* DNA has already been found in archaeological bone remains in Europe and the Middle East [13,14,15,16,17,18,19,20,21], there have been no reports from Far East Asia. This may be related to the geographical and environmental diversity between Europe and Asia, where archaeologists and anthropologists have not paid much attention to palaeopathological topics of leprosy and other infectious diseases [22]; Asia's predominantly hot and humid climate may cause difficulty in recovering skeletons buried for several hundred years.

The present case was excavated in the northern part of Japan and showed typical osteological changes of leprosy. We took skeletal samples from four different areas, the inner surface of the nasal cavity, maxillary palate and diaphysis of the fibula, that showed deformities by erosion and atrophy, or mild periostitis. *M. leprae* DNA was successfully demonstrated in the maxillary palate and fibular diaphysis.

Although subperiostal exostoses and/or hypertrophy accompanied by swelling or porotic hyperosteosis of the fibula are reported as the typical signs of leprosy [6,7], the presence of *M. leprae* DNA in the fibula was somewhat surprising to us because apparent fibular lesion is not common in leprosy patients at present. It is well known that the common fibular nerve is one of the sites preferably affected by *M. leprae*, which causes foot drop in the patient [30]. It may therefore be possible to speculate that *M. leprae* affected the periosteum through this nerve. If that is the case, the present identification of *M. leprae* DNA in the fibula supports the possibility that *M. leprae* can spread significantly throughout the body when patients are left untreated.

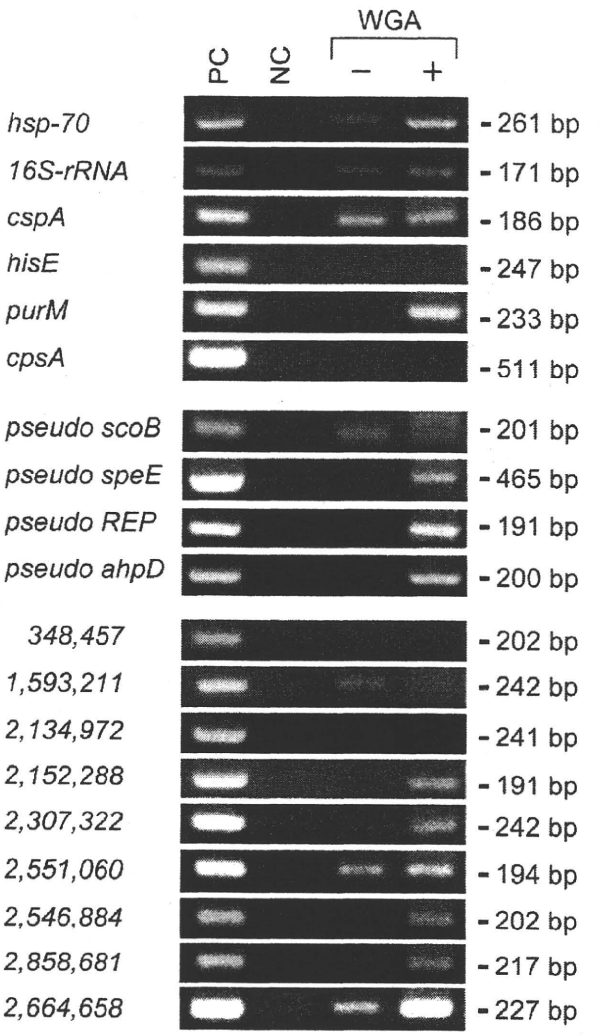

Figure 5. Comparison of conventional PCR and WGA-PCR. PCR was performed using a DNA sample derived from the present study (sample No. 1 shown in Table 1 and Fig. 3). The DNA before and after WGA ("−" and "+", respectively) was amplified using specific nested primers and direct primers targeted for the genes, pseudogenes and non-coding regions of *M. leprae* genome as listed in Table 2. Names of the genes and pseudogenes are indicated and the coordinate positions of non-coding regions in the genome (http://genolist.pasteur.fr/Leproma/) are indicated numerically. PC: positive control DNA from *Thai 53* strain of *M. leprae*; and NC: negative control using DNase/RNase-free water instead of a DNA sample for PCR reaction.
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The SNP genotype of *M. leprae* DNA in *Hatanai* SK26 was type 1, which is a major group in Southeast Asia and India, but rather minor in modern East Asia including Japan and China [2,3]. All reported SNP types of ancient *M. leprae* DNA from Europe and Middle East are type 3, which is also a major genotype in the same regions at present [3]. However, this study confirmed the first case of SNP type 1 as ancient DNA samples. Although more evidence is required, this suggests the possibility that in the Far East inclusive of Japan, the dispersal process of the phylogenetic type of leprosy is historically different from the occidental world. In the Southeast Asia and India where the modern dominant SNP group is type 1, archaeological skeletal remains with lesions of lepromatous leprosy have been excavated and dated from ca. 2000 BC (India) to ca. 300 BC-500 AD (Thailand) [31,32]; therefore the type 1 genotype in modern Japan might be traced back to Southeast Asia and probably originated in India. A historical record of ancient Japan mentioned that the oldest case suggestive of leprosy dated back to the 7th century, and the oldest positive palaeopathological evidence at present is dated to the 13th century. The genotypes of these ancient and medieval materials are still unknown, and further examination of ancient materials from Eastern and Southeast Asia are needed in order to determine the dispersal history of leprosy in Far East Asia.

In our laboratory, PCR detection of *M. leprae* DNA is routinely performed from small amounts of clinical samples of skin smears or paraffin sections as part of a government reference center. It is difficult, however, to apply the same PCR protocol to detect *M. leprae* from ancient DNA recovered from skeletal remains. To overcome this problem, we have utilized WGA, a technique that enables amplification of minute amounts of DNA [29], to increase the sensitivity for PCR detection. We have successfully utilized this method to amplify and detect environmental bacteria from small amounts of water taken from the sea or lakes in Japan (unpublished data).

By PCR reaction using WGA-amplified DNA (WGA-PCR method), large amounts of DNA could be generated from minute amounts of original DNA samples and the detection rate of *M. leprae* DNA and signal intensity were significantly improved. Thus, this method enables highly sensitive detection of small amounts of clinical, environmental and ancient DNA. On the other hand, it also carries the risk of contamination and false positives, particularly if performed by an individual lacking appropriate knowledge and training in molecular biology. Even using this method, however, some primers still did not work on ancient DNA. This might be due to DNA fragmentation in the ancient sample and/or because of the DNA fragmentation process employed during the library preparation step in the WGA protocol. In general, genomic DNA from dead organisms gradually degrades into short fragments over time mainly due to the activity of nucleases produced by microorganisms in the soil. PCR detection, even using the present WGA-PCR method, will be difficult to perform when such a fragmentation proceeds, especially for very old materials and those buried in conditions where the decomposition process is accelerated, *e.g.* a hot and humid environment. Nevertheless, WGA-PCR seems to be advantageous for improving the sensitivity of DNA recovery from these samples. We are now routinely utilizing this method to detect ancient DNA and believe that it will be applicable for detecting other kinds of DNA from archaeological samples, as well as other kinds of samples with trace amounts of DNA.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: KS NI. Performed the experiments: KS WT KT KN YI AK HW SM. Analyzed the data: KS TA MS AY. Contributed reagents/materials/analysis tools: KS TA MS AY. Wrote the paper: KS WT NI.

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Infection during Infancy and Long Incubation Period of Leprosy Suggested in a Case of a Chimpanzee Used for Medical Research[▽]

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The length of the incubation period of leprosy following *Mycobacterium leprae* infection has never been conclusively determined, owing to the lack of a method to demonstrate the presence of an asymptomatic infection. We report a rare case of leprosy in a chimpanzee in which a 30-year incubation period was strongly suggested by single nucleotide polymorphism (SNP) analysis.

CASE REPORT

A female chimpanzee (*Pan troglodytes*) named Haruna was caught in Sierra Leone, West Africa, and brought to Japan in 1980 when she was around 2 years old. She was used in hepatitis B studies for 6 years and in hepatitis C studies for another 4 years before she was retired to live out her life in a sanctuary. She and her fellow chimpanzees were kept according to the guidelines of Chimpanzee Sanctuary Uto, Wildlife Research Center, Kyoto University, Japan. Resident chimpanzees are treated with the same level of care as human patients in hospitals, and all physical examinations were carried out solely for the purpose of diagnosis and treatment, as approved by the sanctuary. Despite her experimental history, she is seronegative for HBsAg, HBs antibody (HBsAb), and HBcAb, as well as for the presence of hepatitis C virus (HCV) RNA. Results of other laboratory tests were all normal.

In January 2009, at around age 31 (the average life span of a chimpanzee is 40 to 50 years and rarely exceeds 50 years), swellings and nodules (Fig. 1A) that had not been observed in the past were noted on her face (Fig. 1B). A thorough examination under anesthesia revealed multiple nodular lesions around the eyes, lips, abdomen, forearms, and crus. Nasal swabs and skin smears from a forearm nodule revealed a large number of acid-fast bacilli, with formation of globi (Fig. 1C and 1D, respectively). Results of a skin tuberculosis (TB) test were negative. Histologic examination of the skin lesion showed a granulomatous accumulation of foamy histiocytes in the upper dermis that contained numerous acid-fast bacilli (Fig. 1E and F) that were positive for PGL-I immunostaining (PGL-I is an antigen specific to *Mycobacterium leprae*). PCR amplification of DNA purified from a skin biopsy sample was positive for *M. leprae*-specific Hsp70 (Fig. 1G), and the DNA sequence of 16S rRNA completely matched the reported *M. leprae* sequence (2).

At this point, a diagnosis of lepromatous leprosy was made and the patient was treated with multidrug therapy (MDT), including diaphenylsulfone, clofazimine, and rifampin, according to the protocol of the World Health Organization (WHO). These drugs were administered after being mixed with bananas or vegetable juice. Within 2 months of treatment, the skin lesions had significantly improved, and within 5 months, nasal swabs tested negative for acid-fast bacilli.

To determine the possible origin of *M. leprae* detected in this case, we analyzed single nucleotide polymorphisms (SNPs) for three reported loci in the *M. leprae* genome (13). By using PCR amplification followed by direct sequencing, positions 14,676, 1,642,875, and 2,935,685 of *M. leprae* DNA were identified as "T," "T," and "C," respectively (Fig. 2). This genotype, which has been identified only in West Africa, was classified as SNP type 4; it is thought to have been introduced to parts of the Caribbean and South America, probably via the slave trade, but has not been identified in Japan or other Asian countries (13, 14).

Increased levels of serum anti-PGL-I antibody have been used for the diagnosis of lepromatous leprosy (1). High levels of serum anti-PGL-I antibody have also been reported in healthy household contacts in an area where the disease is endemic (7). However, there have been some arguments about the specificity of PGL-I for leprosy as well as about the clinical relevance of measuring the level of anti-PGL-I antibody for the diagnosis of leprosy, particularly for the detection of subclinical *M. leprae* infection (9). The results of analysis of archived serum samples from Haruna were negative for anti-PGL-I antibody until 25 October 2007 (ca. 1 year before her skin lesions were observed) and became positive on 13 May 2009, after the appearance of skin lesions, but returned to negative on 8 October 2009, approximately 5 months after MDT was administered (Fig. 3). Eight other chimpanzees imported to Japan in the same year and five others living in the same cage with Haruna for several years were all negative for serum anti-PGL-I antibody.

Leprosy has afflicted humans for millennia and is caused by chronic infection with *M. leprae*. It is believed that the disease

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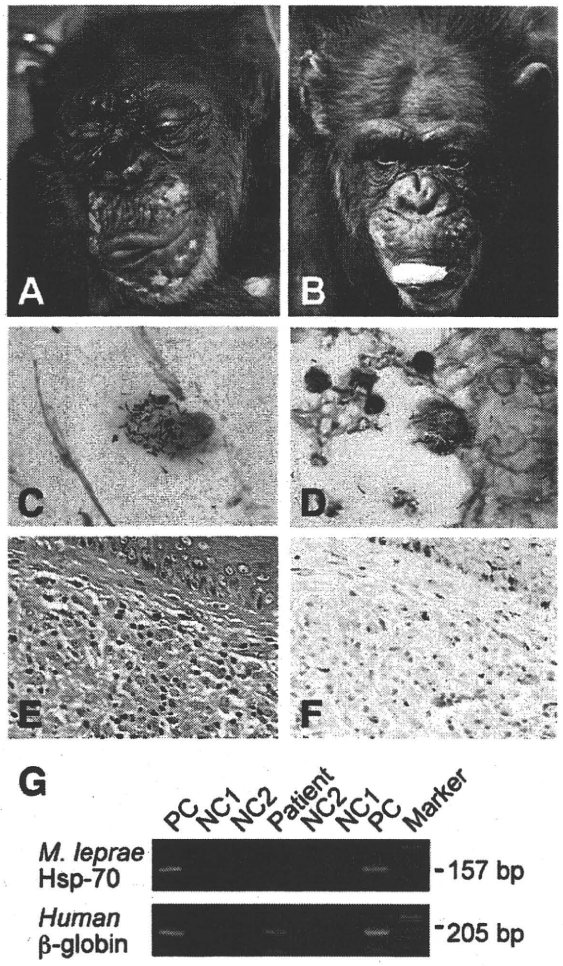

FIG. 1. (A) Facial leproma-like lesions of the chimpanzee Haruna (13 May 2009). (B) The same chimpanzee without lesions 1 year earlier (16 May 2008). (C) Ziehl-Neelsen staining of a nasal swab specimen showing globus-filled acid-fast bacilli (×1,000 magnification). (D) A skin smear from a left forearm nodule also showing multiple acid-fast bacilli (×1,000 magnification). Tissue staining was performed as previously described (18). (E) Hematoxylin and eosin staining of a skin biopsy sample from a right forearm nodule, showing accumulation of foamy histiocytes in the upper dermis (×400 magnification). (F) Fite staining demonstrating numerous acid-fast bacilli within the histiocytes (×400 magnification). (G) PCR analysis demonstrating *M. leprae* Hsp70 DNA. Tissue DNA was prepared using a QIAamp DNA Micro kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. PCR was performed as previously described (18) using specific primers (10, 17). PCR products were sequenced using an ABI Prism 310 genetic analyzer and GeneScan Collection software (Applied Biosystems). PC, positive control; NC1, negative control 1 (nuclease-free water was used as a template for PCR); NC2, negative control 2 (nuclease-free water was used instead of skin tissue to purify DNA). Human β-globin served as a positive control for DNA extraction from the skin biopsy sample.

is primarily transmitted by repeated airborne exposure to *M. leprae* through the nasorespiratory passage via close contact with multibacillary leprosy patients during infancy or early childhood and that the clinical disease becomes apparent only after a long incubation period (3). Although it is thought that *M. leprae* parasitizes histiocytes in the dermis and Schwann

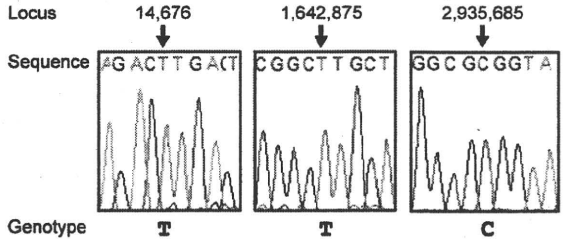

FIG. 2. Sequence analysis of the three reported SNPs of *M. leprae* DNA. PCR was performed using previously described primer sets (13), and the PCR products were sequenced using an ABI Prism 310 genetic analyzer and GeneScan Collection software (Applied Biosystems).

cells of the peripheral nerves (15), there is no available serologic or biologic method to demonstrate the presence of subclinical infection during latency. Therefore, it has not yet been possible to determine the length of incubation following infection.

M. leprae infection in this chimpanzee was highly unlikely to have occurred in Japan, particularly given the strict biosafety standards of primate housing facilities in experimental laboratories and the very low prevalence of leprosy in Japan. Therefore, the evidence strongly suggests that Haruna contracted a *M. leprae* infection when she was in West Africa before the age of 2 and that she developed lepromatous leprosy after a 30-year incubation period. Our results also suggest that the levels of serum anti-PGL-I antibody reflect the bacterial load in the patient but may not represent a marker for subclinical *M. leprae* infection.

To date, only three cases of leprosy in chimpanzees have been reported in the literature. All three chimpanzees were born in Africa and brought to the United States at a young age (4–6). One male chimpanzee captured in Sierra Leone developed leprosy some time between 5 and 6 years of age (4). Gormus et al. suggested the possibility that *M. leprae* might be transmitted among chimpanzees in Africa (5). Others have suggested that contact with an infected human had potentially occurred during the 2-to-3-month period when the chimpanzees were housed in outdoor cages while awaiting shipment after capture (4).

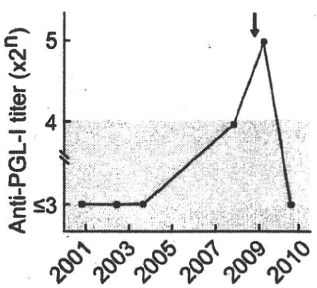

FIG. 3. Changes in the serum anti-PGL-I antibody titer before and after disease onset. The arrow indicates the approximate date when skin lesions appeared. The shaded area indicates the negative range. The serum anti-PGL-I antibody titer was measured using a gelatin particle agglutination test kit, Serodia-Leprae (Fujirebio, Tokyo, Japan), according to the manufacturer's instructions. Antibody titers > 2^5 were judged to represent positive results.

Nonetheless, leprosy is clearly a common disease among humans and apes. Even after the worldwide efforts to reduce the disease burden of leprosy spearheaded by WHO (http://www.searo.who.int/LinkFiles/GLP_SEA-GLP-2009_3.pdf) were successfully completed (8, 16), isolated leprosy cases in chimpanzees or other animals (e.g., armadillos) may still exist in the wild (11, 12, 19) and serve as potential sources of human infection. Therefore, it might be of particular importance to perform a survey of chimpanzee leprosy in West Africa (11).

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Invited Review

Whole-Genome Expression Analysis of *Mycobacterium leprae* and Its Clinical Application

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CONTENTS:

SUMMARY: The whole-genome sequence analysis of *Mycobacterium leprae*, which was completed in 2001, revealed the characteristics of this microbe's genomic structure. Half of the *M. leprae* genome consists of a limited number of protein-coding genes and the rest comprises non-coding regions and pseudogenes. We performed membrane array and tiling array analyses to analyze the gene-expression profile of the *M. leprae* genome and found that pseudogenes and non-coding regions were expressed similarly to coding regions at the RNA level. The RNA expressions were confirmed by real-time PCR analysis. Expression of these RNAs in clinical samples showed varying patterns among patients, thus indicating that the analysis of RNA expression patterns, including non-coding regions and pseudogenes, may be useful for understanding the pathological state, prognosis, and assessment of therapeutic progress in leprosy.

1. Introduction

More than 240,000 new cases of leprosy, a chronic infectious disease caused by *Mycobacterium leprae* that involves the skin and nerves (1), were detected worldwide in 2009 (2). The discovery of its causative agent, *M. leprae*, was made earlier than that of *Mycobacterium tuberculosis*, but it is still impossible to culture this bacillus or to transfer the disease to experimental animals. Although it is known that *M. leprae* preferentially parasitizes macrophages in a foamy or enlarged phagosome filled with lipids (3) and the mechanism by which such a large amount of lipids is recruited and accumulated in phagosomes, and how the expression of host proteins is modified following infection, have been elucidated (4,5), the biological characteristics of *M. leprae* in the phagosome are still largely unknown. Previous studies on the nature of *M. leprae* were limited to methods other than gene manipulation, such as evaluation of respiration or adenosine triphosphate production (6), detection of RNA, and the use of other mycobacteria to study the function of specific *M. leprae* genes (7). The exact nature of *M. leprae* therefore remains unclear.

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2. Advances in the DNA microarray technique

The microarray technique permits the analysis of hundreds of thousands of probes mounted on a glass slide in a single experiment. Recent advances in genome science and the more precise annotation of published genomes for many organisms has enabled probe design for all of the organism's genes and comprehensive analysis of whole-genome RNA expression. Conventional DNA microarray analysis has been applied to various species such as *Escherichia coli* (8,9), *Helicobacter pylori* (10), *Yersinia pestis* (11), and *Salmonella enterica* serovar Typhi (12), as well as to *M. tuberculosis* (13) and *M. leprae* transcriptomes (14,15). However, only known or predicted genes were examined in such arrays, therefore, it was difficult to analyze the RNA expression of non-coding regions and potential pseudogenes that did not have the appropriate annotation and array probes.

The use of tiling arrays makes it possible to design and arrange overlapping probes spanning the entire genome of an organism (16,17). A genome tiling array enables the comprehensive detection of short and long transcripts, including products from non-coding regions, over the entire genome without bias. With typical eukaryote genomes, the tiling array is performed for each chromosome or smaller limited region because of the large genome size (18–20). However, whole-genome tiling array probes for the smaller genomes of prokary-

Table 1. The genome structure of *Mycobacterium* spp.

Mycobacterium spp.	Pseudogene	Gene	% of genome	Genome size (bp)	Reference
M. tuberculosis	6	3,959	90.8	4,411,532	41
M. bovis	27	3,953	90.6	4,345,492	56
M. marinum	65	5,424	90.0	6,636,827	57
M. smegmatis	168	6,897	92.4	6,988,209	58
M. ulcerans	771	4,160	72.3	5,631,606	28
M. leprae	1,116	1,605	49.5	3,268,203	26

otes and fungi have been designed (21) and applied in various species such as *E. coli* (22), *Bacillus subtilis* (23), *Neisseria meningitidis* (24), and *Saccharomyces cerevisiae* (25).

3. Characteristics of the *M. leprae* genome

It is thought that the high degree of host dependency of *M. leprae* is related to its genetic characteristics. The whole-genome sequence of *M. leprae*, which was published in 2001, revealed that only half of the small genome contains protein-coding genes, while the remainder consists of pseudogenes and non-coding regions (26). The number of pseudogenes in the *M. leprae* genome is much larger than in other mycobacteria (Table 1) (27). *Mycobacterium ulcerans* has the second-highest number of pseudogenes, 771, although the proportion of pseudogenes based on genome size is less than half that of *M. leprae* (28). The number and proportion of pseudogenes in *M. leprae* are also exceptionally large in comparison with other pathogenic and non-pathogenic bacteria and archaea (29,30). It has been shown that many *M. leprae* pseudogenes are caused by insertions of stop codons (31), and this is speculated to be caused by the dysfunction of sigma factors (32) or the insertion of repetitive sequences derived from transposons, which comprise up to 2% of the *M. leprae* genome (33). Despite such genetic damage, specialized intracellular environments that are free from evolutionary competition allow its survival (26,34,35). Moreover, the proportion of non-coding regions in the *M. leprae* genome (24%) is also greater than that of *M. tuberculosis* (9%). It has been speculated that *M. leprae* has lost over 1,500 genes from its complete genome set and that non-coding regions are functionally silent and useless "junk DNA" (36). However the expression patterns of non-coding regions have never been analyzed, therefore the functional roles of these unique pseudogenes and non-coding regions are unknown.

4. Detection of pseudogene expression by membrane array

In the course of studying the gene-expression profile of *M. leprae* upon infection, we found that some *M. leprae* pseudogenes are highly expressed as RNA (37). In this experiment, genomic DNA fragments prepared from the *Thai*-53 strain of *M. leprae* were cloned into shuttle cosmid vectors for *E. coli* and mycobacteria to construct a genomic DNA library. Clones overlapping in sequence and covering 98% of the *M. leprae* genome (137 clones) were selected and each cosmid was spotted onto a nylon filter. Messenger RNA was purified from *M. leprae* with or without infection into macrophages, then each mRNA was enriched by cDNA subtraction. Radiolabeled cDNA was hybridized using the membrane array technique (Figs. 1A and B) and the signal intensities determined (Fig. 1C). Eight cosmid clones whose expression was significantly modified by infection were digested with eight different restriction enzymes; Southern blot analysis was then performed using the same probe used for the membrane array (Fig. 1D). The location of each hybridized fragment was analyzed in silico by comparison with the restriction map and gene annotations of the *M. leprae* genome. As a result, 12 genes, including six pseudogenes, were identified as genes whose expression level was high and modulated by infection. The high expression level of the pseudogenes suggests that they may have some functional role, such as antisense activity (38), and that they might be involved in infection and/or intracellular parasitization.

5. Detection of RNA expression by whole-genome tiling array

We then designed a whole-genome tiling array to analyze the comprehensive RNA expression of genes, pseudogenes, and non-coding regions in *M. leprae* (39). Since shorter probes achieve higher resolution but result in low signal specificity, the probe length was set to 60-mer and the adjacent probe was shifted by 18 nucleotides (42-nt overlaps). A total of 363,116 probes were designed for the sense and antisense strands based on sequences obtained from the GenBank database (accession no. NC_002677) (26). For this genomic tiling array, *M. leprae* RNA from SHR/NCrj-*rnu* hypertensive nude rats (40) was labeled and hybridized, and the fluorescent signal intensity was normalized. The signal-intensity pattern for each probe was found to be similar to that obtained from an *M. leprae* ORF array, which was hybridized using the same RNA sample, thereby confirming the specificity of the tiling array. The strongest signal was identified in the rRNA, with most probes in this region showing significantly higher intensity (Fig. 2A). Other highly expressed areas were also detected in the gene (Fig. 2B), pseudogene (Fig. 2C), and non-coding regions (Fig. 2D).

For this array, highly expressed regions were defined as areas where the intensities of four probes within 500 bp were higher than 60% of the maximum intensity. As a result, a total of 209 highly expressed regions were determined from gene, pseudogene, and non-coding regions based on annotation (Table 2). In this study, non-coding regions were defined as regions that are not

annotated. Surprisingly, the RNA expression from non-coding regions was found to be the highest in terms of number, mean length, and mean peak signal intensity, whereas that from genes (ORFs) was statistically the lowest. RNA expression from 18 of the 209 highly expressed regions was detected by RT-PCR and real-time PCR.

Gene and pseudogene expression profiles were obtained from the tiling array of the *M. leprae* genome. Since *M. leprae* genes and pseudogenes are functionally classified into six groups (26,41), the highly expressed genes were classified according to these same criteria (Table 3). *M. leprae* grown in hypertensive nude rats was found to have relatively high expression levels in the "cell processes" class, which is related to the folding of synthesized proteins like chaperones and transporters (9.8%) (42), whereas a low expression ratio was found for the pseudogenes (3.0%). This pattern is consistent with the proposal that genes in this class play very essential roles. Furthermore, a high degree of expression was detected for lipoproteins and the PE and PPE families (3 out of 11) in the "other functions" class. The PE and PPE families are specific to *Mycobacterium* spp. (41) and are associated with the early secreted antigenic target 6-kDa (ESAT6) antigen (43), therefore they may play an important role in virulence, as exemplified in *M. tuberculosis* (44). Because *M. leprae* has fewer PE, PPE, and ESAT6-like genes than *M. tuberculosis*, information on these expressed genes will facilitate further functional analysis of a PE, PPE and ESAT6-like protein complex.

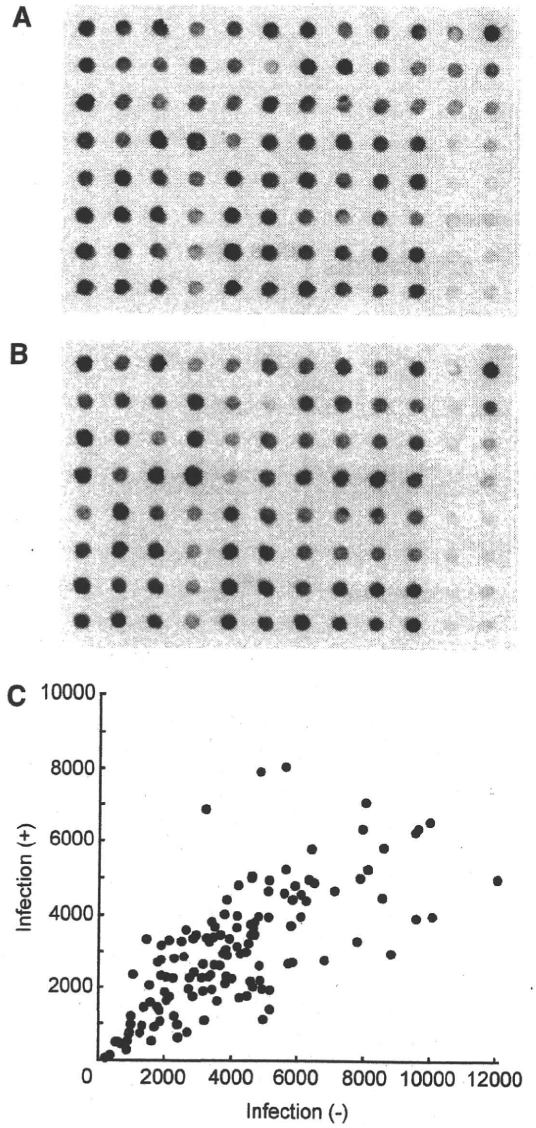

Fig. 1. Hybridization of *Mycobacterium leprae* genome arrays with ^{33}P-dCTP-labeled cDNA probes. *M. leprae* DNA covering over 98% of the genome was purified from a cosmid library, spotted on membranes and probed with cDNA from bacilli-infected macrophages (A) or cDNA from control bacilli (B) after subtractive enrichment. (C) Correlation of signal intensity from arrays hybridized with subtracted cDNA. Densitometric analysis of signal intensity was performed on the array hybridizations and results from the same clones were compared. The *y* axis denotes the results from bacilli-infected macrophages, as shown in Fig. 1A. The *x* axis denotes results from the control bacilli shown in Fig. 1B. (D) Identification of fragments containing differentially expressed cDNAs. The cosmids demonstrating significant differences in signal with or without infection were digested with eight restriction enzymes and separated on a 1% agarose gel (left panel) with marker DNA (leftmost lane). Gels were subsequently transferred to nylon filters and hybridized with the same probes that were used for dot blot hybridization (right panel).

6. Clinical application of *M. leprae* RNA analysis

There is currently no sensitive marker to reflect the disease type or clinical course of leprosy, therefore we decided to analyze clinical specimens to see how the mRNAs were expressed in *M. leprae* from infected patients. PCR primer sets were designed against expressed regions in the *M. leprae* genome and RT-PCR was performed on *M. leprae* RNA extracted from skin smear samples from lepromatous leprosy patients (45). The results showed that the RNA expression patterns were quite different among the patients, although *M. leprae* Hsp70 was detected in all specimens examined (Fig. 3). The differences in pseudogene and non-coding region expression suggest that these may have some functional role and that their expression may be related to disease type, clinical course, or treatment (46). Although further studies are needed, it should be possible to establish an RT-PCR-based diagnostic panel to rapidly diagnose the effects of drug therapy, disease type, and lepra reaction.

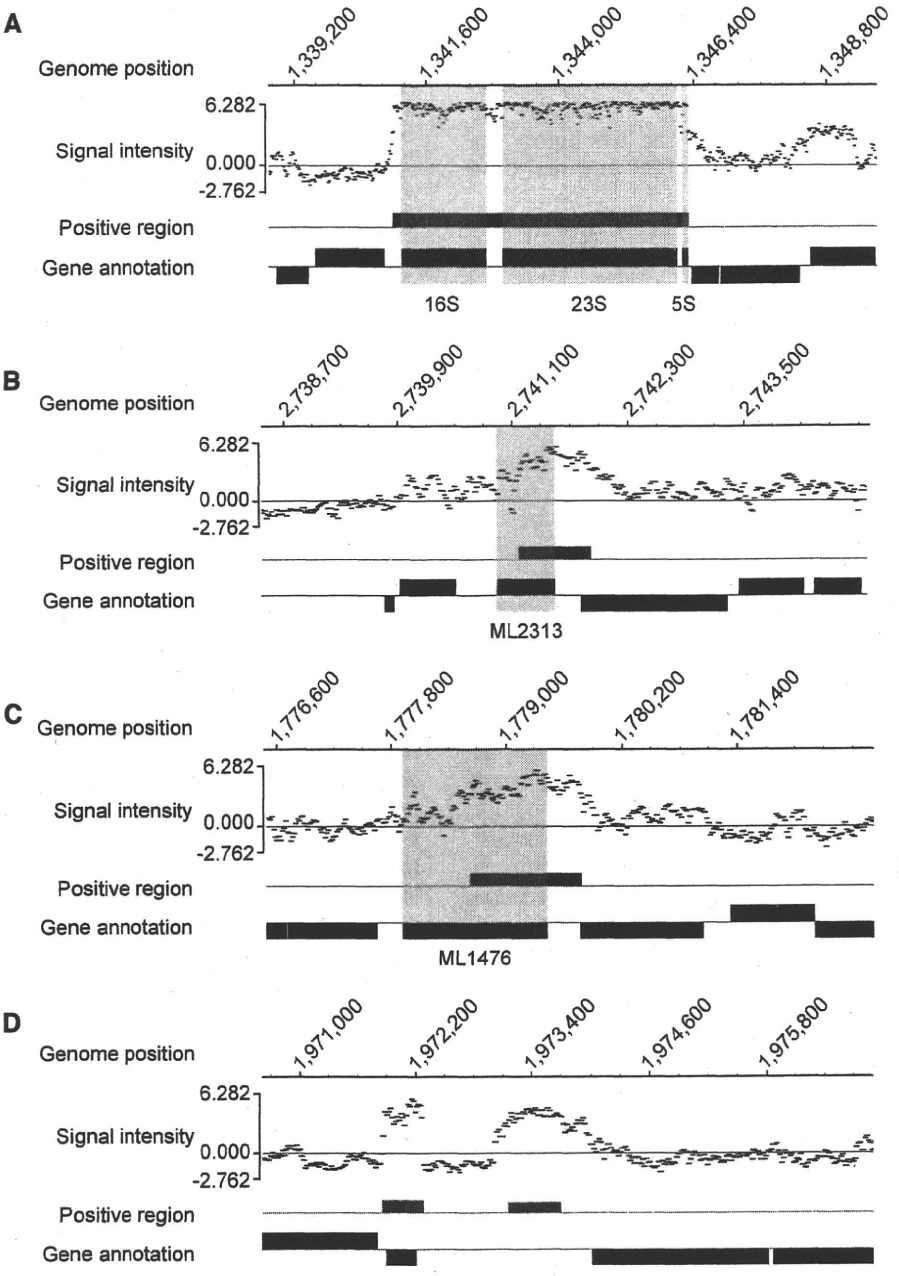

Fig. 2. Signal intensity patterns detected as highly expressed areas in the tiling array. Each probe was arrayed against the corresponding *M. leprae* genome sequence. Positive areas were extracted and are depicted under the signal pattern of probes with gene and pseudogene annotations. (A) Genomic region of rRNA showing nearly saturated signal intensity. (B) Highly expressed region of the gene for the ML2313 hypothetical protein (shaded area). (C) Highly expressed region of the pseudogene ML1476, pseudo-probable oxidoreductase alpha subunit (shaded area). (D) Highly expressed non-coding region in genomic position 1,973,155 to 1,973,700 bp, which showed no homology to genes or other functional sequences by BLASTN search.

Table 2. Number of highly expressed genes, pseudogenes, and non-coding regions identified by tiled microarray analysis

	Gene	Pseudogene	Non-coding region	Total
No. identified	63	78	68	209
% of total	30.1	37.3	32.5	100
Mean length (bp)	637	611	634	—
Mean peak intensity[1)]	4.88	5.11*	5.38**	—

[1)]: Mean peak intensity of pseudogenes and non-coding regions were statistically compared with the intensity of coding genes (*, $P < 0.05$; **, $P < 0.00001$ by Student's t test).

7. Conclusion

We have been able to detect strong RNA expression from pseudogenes and other non-coding regions by using an *M. leprae* genome tiling array. Although the biological function of the RNA transcribed from these regions is unknown, short RNA fragments generated from non-coding regions have been found in many organisms (47). It is known that most of the microRNAs detected in the transcriptome of eukaryotes are non-coding RNAs (48). Moreover, it is reported that pseudogenes also have some biological functions in proc-

Table 3. Number and percentage of expressed genes and pseudogenes based on functional classification (26,41)

Classification	Gene[1)]	Pseudogene[1)]
Small-molecule metabolism (Synthesis and degradation of amino acid, polyamine, nucleotide, cofactor and lipid, energy metabolism enzymes)	19/467 (4.1%)	19/334 (5.7%)
Macromolecule metabolism (Synthesis and degradation of protein, RNA, DNA, and cell envelope)	16/458 (3.5%)	10/163 (6.1%)
Cell processes (Transporter and chaperon)	10/102 (9.8%)	2/67 (3.0%)
Other functions (Virulence, repeated sequence, PE and PPE families)	6/77 (7.8%)	29/133 (21.8%)
Conserved hypotheticals	6/360 (1.7%)	18/416 (4.3%)
Unknown	6/141 (4.3%)	0/2 (0%)
Total	63/1605 (3.9%)	78/1115 (7.0%)

[1)]: The ratio is the number of expressed genes or pseudogenes/ total number of genes or pseudogenes.

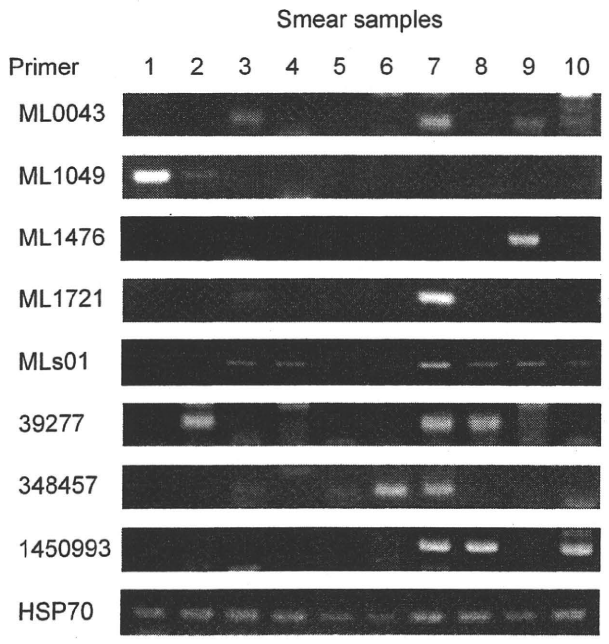

Fig. 3. RT-PCR analysis of *M. leprae* RNA derived from skin smear samples from 10 lepromatous leprosy patients. Total RNA was isolated from skin smear samples. After treatment with DNase I, RT-PCR was performed for pseudogenes ML0043, ML1049, ML1476, ML1721, non-coding genomic region positions 39277, 348457, 1450993 with known non-coding RNA MLs01, and Hsp70.

esses such as cell growth and organogenesis (49), or cancer and the central nervous system (50), through the regulation of gene expression (51,52). Although the proportion of non-coding regions in microbes, as well as their genome sizes, is much smaller than for eukaryotes, RNA expression from non-coding regions was also been detected from the whole-genome analysis of *E. coli* (53) and *Prochlorococcus* and *Synechococcus* (54). Tiling array analysis also identified novel non-coding RNAs in yeast (55). Since exceptionally large numbers of pseudogenes and non-coding regions were shown to be expressed as RNAs in *M. leprae*, this may prove to be a useful model organism for the study of the molecular mechanisms underlying the creation and the role of these transcripts.

The diagnosis of leprosy primarily relies on clinical and microscopic examination. It is difficult to predict the efficacy of treatment or the occurrence of lepra reactions. Sensitive and feasible molecular analyses should therefore provide important information for clinical diagnosis. In particular, it is possible that the analysis of *M. leprae* RNA expression will provide a method to assess the efficacy of multidrug therapy. Further analysis of large numbers of clinical specimens will hopefully enable the construction of an RT-PCR-based diagnostic panel.

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Conflict of interest None to declare.

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RESEARCH LETTER

Biochemical characteristics among *Mycobacterium bovis* BCG substrains

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BCG; substrain; vaccine; biochemical characteristics; tuberculosis.

Abstract

In order to evaluate the biochemical characteristics of 14 substrains of *Mycobacterium bovis* bacillus Calmette Guérin (BCG) – Russia, Moreau, Japan, Sweden, Birkhaug, Danish, Glaxo, Mexico, Tice, Connaught, Montreal, Phipps, Australia and Pasteur – we performed eight different biochemical tests, including those for nitrate reduction, catalase, niacin accumulation, urease, Tween 80 hydrolysis, pyrazinamidase, *p*-amino salicylate degradation and resistance to thiophene 2-carboxylic acid hydrazide. Catalase activities of the substrains were all low. Data for nitrate reduction, niacin accumulation, Tween 80 hydrolysis, susceptibility to hydrogen peroxide and nitrate, and optimal pH for growth were all variable among these substrains. These findings suggest that the heterogeneities of biochemical characteristics are relevant to the differences in resistance of BCG substrains to environmental stress. The study also contributes to the re-evaluation of BCG substrains for use as vaccines.

Introduction

Biochemical tests are currently used as a technique for the identification of bacterial species. Recently, several studies have investigated the physiological meaning of the biochemical characters in the genus *Mycobacterium*. Sohaskey and colleagues reported variable nitrate production among *Mycobacterium bovis* bacillus Calmette Guérin (BCG) substrains in relation to survival in host cells (Sohaskey, 2008; Sohaskey & Modesti, 2009). Recycling of NAD and NAD-quinoline reductase relevant to the latent infection of *Mycobacterium tuberculosis* and resistance to oxidative stress, respectively, have also been reported (Boshoff *et al.*, 2008). Mycobacterial phospholipase A (MPLA) catalyses the hydrolysis of lipids including Tween 80 (Parker *et al.*, 2007), and this activity appears to contribute to survival under starvation at the dormant stage of growth (Jackson *et al.*, 1989; Deb *et al.*, 2009). Here, we analysed the biochemical characteristics and their relationship to susceptibility to environmental stress, such as oxidative stress, nitrosative stresses and pH changes, among BCG substrains.

Materials and methods

Bacterial strains

Mycobacterium bovis BCG strains Australia (ATCC 35739), Birkhaug (ATCC 35731), Connaught (ATCC 35745), Danish (ATCC 35733), Glaxo (ATCC 35741), Mexico (ATCC 35738), Montreal (ATCC 35735), Pasteur (ATCC 35734), Phipps (ATCC 35744), Tice (ATCC 35743), Russia (ATCC 35740) and *M. tuberculosis* strain $H_{37}Rv$ (ATCC 25618) were purchased from American Type Culture Collection (ATCC, Manassas, VA). BCG-Moreau, *M. bovis* (JATA) and *Mycobacterium smegmatis* were provided by Dr M. Takahashi (The Research Institute of Tuberculosis Japan Anti-tuberculosis Association, Kiyose, Tokyo, Japan). BCG-Japan (Tokyo 172) was purchased from Japan BCG Laboratory (Kiyose, Tokyo, Japan). BCG-Sweden (vaccine seed) was provided by Dr S. Yamamoto (Japan BCG Laboratory). *Mycobacterium avium* strains 724S and 2151SmO were kindly provided by Drs J. Inamine and E. Torsten (Colorado State University, Fort Collins, CO).