ORIGINAL ARTICLE

AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY SEPTEMBER 2009 ISBN 1595-689X VOL 10(3)

AJCEM/200908/20921 -http://www.ajol.info/journals/ajcem

AFR. J. CLN. EXPER. MICROBIOL 10(3): 185-199

THE USE OF RAP-PCR IN STUDYING *MYCOBACTERIUM TUBERCULOSIS* INTRACELLULAR GENE DURING MACROPHAGE INFECTION.

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Running title: *RAP-PCR* in studying *Mycobacterium tuberculosis* intracellular gene expression

ABSTRACT

***Mycobacterium tuberculosis* is the second leading cause of death from infectious agent. This study sought to detect *M. tuberculosis* genes, which were specifically expressed, or upregulated during intracellular infection of J774 murine macrophages; as such genes may be potential targets for novel drug action. J774 murine macrophage cell line was infected with *M. tuberculosis* (H37Rv strain) at 10:1 multiplicity of infection (MOI). RNA was differentially extracted from *M. tuberculosis* infecting J774 macrophage cell line. The control in this case was RNA from extracellular broth grown bacteria. Approximately 50 ng of RNA from intracellular derived bacteria and extracellular derived bacteria (control) were subjected to random arbitrarily primed PCR (RAP-PCR) using 50 primer combinations. Eleven differential RAP-PCR products were observed. All RAP-PCR products were cloned into pCR®2.1 and sequenced in order to determine the identity of the products. Four of the eleven products were derived from macrophage genes and another 4 products were derived from the *M. tuberculosis* rRNA genes (three 23S and one 16S rRNA). The 3 remaining RAP-PCR products were found to be mycobacterial genes other than ribosomal genes. The three products were genes encoding enzyme involving in a shikimate pathway, a putative carboxyphosphonoenolpyruvate phosphonomutase and a serine protease with homology to HtrA. Of the 3 mycobacterial genes other than ribosomal genes detected, none were specifically expressed during intracellular infection but competitive RT-PCR showed that *aroF* gene was upregulated two-fold in intracellular derived bacilli.**

Keywords: - *RAP-PCR/Mycobacterium tuberculosis/* macrophage infection/RAP-PCR

INTRODUCTION

Mycobacterium tuberculosis is the second leading cause of death from any infectious agents in developed and developing countries after deaths due to human immunodeficiency virus (HIV), accounting for one-third of the mortality due to infectious. Over the past few years, there has been an increased in the incidence of tuberculosis partly due to the HIV infection pandemic [1]. About 9.2 million people was estimated to be new cases of tuberculosis (TB) worldwide in 2006 – an increase of 0.6 % from 2005 with 1.7 million died from the disease [2]. The emergence of multi-drug resistant strains of *M. tuberculosis* [3] also complicated the treatment of this infection. In addition, there is no adequate vaccine for prevention of the disease. The BCG vaccine - a

vaccine of choice in most of the countries has variable degree of vaccine efficacy ranging from 0 to 90% [4-6]. Therefore, there is need for new intervention in terms of new chemotherapeutic agents and design of effective vaccines. Design of new chemotherapeutic agents and vaccines require better understanding of the basic biology of the organism including the metabolism. How *M. tuberculosis* survives the early interaction with the alveolar macrophages still remains a mystery. Understanding the survival mechanisms of *M. tuberculosis* could only come from the use of molecular biological techniques. Many of the molecular biological techniques require the use of a well-developed genetic system for mycobacteria. Progresses have been made in this area over the last few years with the development of transposon mutagenesis [7], homologous recombination [8], and *in vivo* complementation test [9, 10]. The difficulty in applying some of these techniques called for the use of other techniques that do not rely on a well-developed genetic system such as RNA based approach. The expression of a gene is indicated by the transcription of a particular segment of DNA into RNA representing the template carrying the necessary information for protein synthesis. RNA synthesis is in a continuous state of flux, which could be a reflection of environmental changes or demand for a particular gene to carry out a specific function. Plum and Clark-Curtiss (1994) had used RNA-cDNA subtractive hybridisation to identify a macrophage induced gene that was expressed by *M. avium* during tissue culture infection [11]. Another approach using the RNA based approach is arbitrary-primed polymerase chain reaction (AP-PCR) or differential display reverse transcription PCR (DDRT-PCR). AP-PCR or DDRT-PCR developed by Liang and Pardee (1992) is based on the use of random primers to identify differences in gene expression between two target cell populations [12]. Wong and McClelland (1994) used this approach to study differential gene expression when *S. typhimurium* was exposed to oxidative stress (H_2O_2) [13]. Kwaik and Pederson (1996) had used a similar technique to identify a gene specifically expressed (*eml* – early stage macrophage-induced locus) by *Legionella pneumophila* during macrophage residency [14]. RNA extraction techniques have improved in mycobacteria over the last few years [15, 16], which makes it feasible for anybody who wants to study gene expression via transcription This study was aimed at using RAP-PCR to detect genes that are differentially expressed by *M. tuberculosis* during the intracellular infection of macrophages.

MATERIALS AND METHODS

Maintenance and culture of mycobacteria.

Mycobacteria species used in this study was *M. tuberculosis* H37Rv (ATCC 9360) obtained from the National Culture Type Collection, Colindale, UK. Mycobacteria were grown to mid log phase in Middlebrook 7H9 broth (Difco Laboratories Ltd., Surrey, UK) supplemented with 10% albumin-dextrose catalase enrichment (ADC; Difco) and 0.02% Tween 80 (Sigma, Dorset, UK) at 37^{o}C (in the presence of 5% CO_2 for *M. tuberculosis)* before harvesting at 0.5-1.0 OD_{600}. *M. tuberculosis* H37Rv cultures were also grown for 3 weeks at 37^{o}C on thick 7H10 agar plates supplemented with 10% oleic acid-albumin-dextrose catalase enrichment (OADC; Difco) and 0.2% glycerol and then stored at 4^{o}C.

Maintenance and culture of macrophage cell line.

The murine macrophage cell line J774.2 (obtained from European Collection of Cell Cultures, CAMR, Porton Down, Salisbury, UK) was used in all infection experiments. J774.2 macrophages were cultured in

Dulbecco's modified Eagle's medium (DMEM) containing 5% heat-inactivated foetal bovine serum (Life Technologies, Paisley, UK) with no antibiotic supplements. Macrophage monolayers were maintained at 37°C in humidified air containing 5% CO_2 before and after infection. For storage purposes, cell pellets were suspended in 9% dimethyl sulphoxide (DMSO) in foetal bovine serum (FBS) at a concentration of approximately 4×10^6 cells/ml and aliquoted into 1 ml cryo-vials (Nunc, UK). The vials were placed in a polysytrene box and then placed at -20°C overnight before transferring to liquid nitrogen.

Macrophage infections and preparation of RNA.

J774.2 macrophages were synchronously infected overnight with non-opsonised static cultured of 10:1 *M. tuberculosis* bacilli in mid log phase [17]. After 4 hr the medium was discarded and washed in three changes of Hanks' balanced salt solution (HBSS) to remove the extracellular bacilli before the infected cell line layer was resuspended in DMEM. A control comprised *M. tuberculosis* bacilli growing in Middlebrook 7H9/ADC/Tween 80 broth without shaking. RNA was prepared from macrophages infected with *M. tuberculosis* using a method based on a differential lysis after overnight incubation. Briefly, the culture medium was discarded and the infected macrophage monolayer was re-suspended in 25 ml guanidine thiocyanate (GTC) solution per flask to lyse the macrophages. In order to reduce the viscosity of the solution, a long thin nosed plastic Pasteur pipette was used to squirt the lysate solution in and out with force to shear the macrophage nucleic acids. The lysates were transferred to 30 ml sterile universal tubes and centrifuged at 2,500×g for 20 min to concentrate the intracellular bacilli. The pellets of intracellular bacilli were combined using 1 ml of wash solution (1 ml 0.5 % Tween 80) and transferred to a 1.5 ml Eppendorf tube and centrifuged in a microcentrifuge at 12,000×g for 30 sec. The supernatant (wash solution) was saved and placed in a fresh Eppendorf tube and stored at -80°C. The pellet of bacteria was re-suspended in 200 µl of sterile DEPC treated water. The RNA was extracted from the harvested intracellular bacilli and the extracellular bacilli control using Mangan et al's method [16]. All RNA samples were DNase I (Pharmacia, UK) treated to destroy any contaminating DNA by incubating the RNA samples in the presence of the enzyme for 30 min.

RAP-PCR.

Two different concentrations of the RNA (approximately 50 ηg and 25 ηg) extracted from the intracellular bacilli and the controls (extracellular bacilli control and the saved Tween 80 wash from the intracellular bacilli) were reverse transcribed in a 20 µl reaction volume containing 0.5 µM of arbitrary primer (15-20-mer) with 0.5 mM each of dATP, dGTP, dCTP and dTTP with 100 U Superscript™ II RNase H- reverse transcriptase (Invitrogen) in the presence of 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol (DTT) following the manufacturer's instructions. Briefly, the RNA sample and the arbitrary primer were incubated together for 15 min at 70°C and chilled on ice before the addition of dNTPs. This was incubated for 2 min at 42°C. Thereafter, reverse transcriptase was added. The reaction mixes were first incubated at 25°C for 15 min to allow for non-specific hybridization and finally incubated for 50 min at 42°C to allow for cDNA synthesis. Following cDNA synthesis, the reverse transcriptase was denatured for 15 min at 70°C. One tenth of the cDNA representing 2 µl was amplified in a 20 µl PCR reaction volume containing 0.5 µM of arbitrary primer (15 – 20 mer), 10 mM Tris-HCl (pH

8.3), 1.5 mM $MgCl_2$, 50 mm KCl, 0.01% gelatin, and 62.5 μM of dNTPs, and 1 U of *Taq* polymerase. The PCR parameters used were denaturation (94°C, 30 sec), annealing (35°C, 1 min), and extension (72°C, 2 min) for 45 cycles in a Perkin-Elmer Gene Amp 9600 thermal cycler. With RAP PCR strategy, 50 primer combinations were used. Following RAP-PCR, 7 μl portions of the RAP-PCR products were added to 2 μl of sample loading buffer and the samples were electrophoresed along with a 100 bp DNA ladder marker through a Clean gel using the Multiphor system. Following electrophoresis, the gel was silver stained. Differential RAP-PCR products were excised from the gel. The excised DNA bands were eluted and re-amplified as before. The reproducibility of the RAP product was re-assessed using the same cDNA, new cDNA preparation from the same RNA samples and also cDNA from independent infection experiment prior to cloning and sequencing.

Cloning and Sequencing.

RAP products were re-amplified using the same cycling parameters which generated the original product except that the final extension step at 72°C was increased to 10 min to provide TA overhangs. The re-amplified RAP product was cloned using TA cloning kit (Invitrogen, The Netherlands.) into a plasmid cloning vector (pCR2.1) following manufacturer's instructions. Plasmid DNA was extracted using the method of Birnboim and Doly [18]. PCR was used to screen the plasmid for the cloned RAP product and the plasmid containing the desired RAP product was used in sequencing. Sequencing was undertaken using the ABI PRISM™ dye terminator cycle sequencing kit (Applied Biosystems) following manufacturer's conditions. The sequencing products were sent to Alta Bioscience, University of Birmingham, Birmingham, UK for analysis.

Computer analysis of sequence data.

Nucleotide sequences were analysed using the University of Wincosin's Genetics Computer Group (GCG) software package [19] on the UNIX system of the University of Birmingham Bioinformatics Unit. The BLAST programs [20] were carried out at the National Institute for Biotechnology Information, National Institute of Health, The Institute of Genetic Research, USA and Sanger Centre, Cambridge, UK.

Quantitative Competitive RT-PCR.

RNA (0.5 μg) was reverse transcribed in a 20 μl containing 0.5 μM of downstream primer AROFB (5'-GGATCACATGCCGTCTCATA-3') for *aroF* gene , 50 mM Tris-HCl pH 8.3; 75 mM KCl; 3 mM $MgCl_2$ and 10 mM DTT with 10 units of Superscript RNase H- reverse transcriptase. The reaction was incubated at 42°C for 50 min. The reaction was stopped by incubating at 70°C for 15 min. Quantitative RT-PCR was performed using the method described by Celi *et al* [21] in preparing the competitive template. In this assay, the concentration of the cDNA was adjusted based on 16s cDNA relative concentration and fixed in a competitive PCR containing series of two fold dilution of the competitive template for *aroF*, 15% glycerol, 0.125 mM of dNTP, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1 μM AROFA (5'-GTCACTTCGATCGCATTGTC-3') and 1 μM AROFB (5'-GGATCACATGCCGTCTCATA-3'). The cycling parameters used are as following: 94°C - 5 min for 1 cycle; 94°C - 30 s; 50°C - 30 s; 72°C - 30 s for 35 cycles and 72°C - 10 min. for 1 cycle. The expected product sizes for the competitive and cDNA amplicons were 120 bp and 180 bp, respectively.

RESULTS

The RNA extracted from the bacterial pellets represented majority of the intracellular *M. tuberculosis* following selective removal of macrophage nucleic acids as indicated by the presence of 16S and 23S bands on agarose gel (data not shown). Using RAP-PCR, eleven products were identified of which three RAP products were identified from *M. tuberculosis* (H37Rv) during intracellular infection that appeared not to be present in the extracellular bacilli (mycobacteria grown in Middlebrook's medium) using 50 primers combination. Four out of the eleven were macrophage genes with another 4 RAP products being mycobacterial ribosomal RNA genes (three 23s rRNA and one 16s rRNA genes). The three remaining RAP products were found to be mycobacterial genes apart from the ribosomal genes earlier mentioned. The three RAP Products were designated RAP 61, RAP 136 and RAP 148. The first RAP product - RAP61-1 (figure 1) was found to be about 400 bp. Two hundred and seventy bases were sequenced and subjected to computer analysis. The DNA search on NCBI using the BLAST programme showed that the DNA has homology to a gene on *M. leprae* cosmid B1559 ($p=9.2e^{-63}$) and also to SEC101 gene of *Streptomycess lividans* ($p=2.1e^{-36}$). At amino acid level using the BLASTX programme, the gene was found to show strong homology to other *aroF* from plants and other bacteria. Apart from this particular gene, it also showed homology to phenazine F of *Pseudomonas aureofaciens* ($p=7.0e^{-06}$). The gene annotation in Sanger centre database showed the gene coded for a product with 462 amino acid residues with strong homology to *aroF* gene of plants. The *aroF* gene has 48% identity over 444 amino acid residues with the *aroF* of *Helicobacter pylori,* 90% identity to aroF of *M. leprae* over 462 amino acid residues, 50% identity to *Arabidopsis thaliana* over 436 amino acid residues and 43% identity to phenazine F of *Pseudomonas aureofaciens* over 429 amino acid residues. The sequence alignment of the putative AroF in figure 2 shows how related the AroF of *M. tuberculosis* to AroF of plants and bacteria.

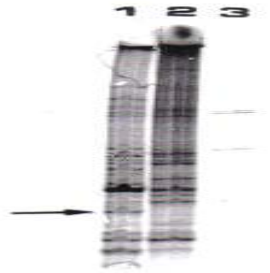

Figure 1. An autoradiograph of the ^{33}P labeled RAP-PCR products derived from cDNA of intra- Cellular *M. tuberculosis* after 15 hr of infection of J774.2 macrophage like cells (lane 1), *in-vitro* grown M. tuberculosis (lane 2) and from J774.2 macrophage (lane 3). The arrowhead shows the RAP-61 whose RAP-PCR product was sequenced and found to be *aroF* gene.

The second differential RAP product - RAP 136-9 (data not shown) was found to be about 300 bp in size. Sequencing result showed that this gene has homology with a gene on MTCY39 (p=0.0) using the FASTA program on GCG at DNA level. The function of the gene is not known but it has homology with carboxyphosphonoenolpyruvate phosphonomutase gene of *Streptomyces hygroscopicus* (p=0.17) and unknown protein of *Saccharopolyspora erythraea* ($p=1.9e^{-56}$) at amino acid level using BLASTP progamme at NCBI. This unknown protein of *Saccharopolyspora erythraea* has been implicated in the final hydroxylation step in erythromycin biosynthesis [22]. Carboxyphosphoenolpyruvate

phosphonomutase has been characterised *hygroscopicus* SF1293 [23]. The general clue that we could derived from the result of the database search is that this gene might be involved in antibiotic synthesisas an enzyme catalyzing the formation of an unusual C-P bond that is involved in the biosynthesis of the antibiotic bialaphos (BA) in *Streptomyces*.

The third differential RAP-145 (data not shown) obtained using this approach with new primers set gave about 200 bp in size. The sequencing result showed that this product was found to have 100% homology to a cosmid MTCY16A12 on searching the *M. tuberculosis* DNA database at Sanger centre. The whole sequence (about 6,400 bp) of the cosmid was pulled out for further analysis at BCM using the program called BCM gene finder. Using this program the RAP 145 was within one of the two potential genes on the cosmid. The putative translated sequence of the gene which was 464 amino acids was subjected to further computer analysis to find the likely identity of the gene on the gene database at NCBI. This gene was found to show homology to most of the high temperature response (*htrA*) genes from other bacteria *including M. tuberculosis* and *M. leprae htrA* gene.

The degree of homology to the *htrA* gene of the *M. tuberculosis* was not 100% suggesting that this gene is not *htrA* but has similar properties to the *htrA* gene. At the upstream of the *htrA*-like gene was the two component regulatory gene with homology to other two component regulatory genes in the GENBANK database. The *htrA*-like gene on Sanger's database is referred to as serine protease gene. Further computer analysis revealed that this protein is a membrane bound protein as indicated by the presence of the myristotyl domain.

To confirm that genes detected by RAP-PCR were differentially expressed a PCR based strategy had to be developed because of the small amount of RNA obtained from intracellular bacilli (typically, 20 ng from a flask of infected macrophages) negating the possibility of using Northern analysis or RNAse protection assays to confirm upregulation of gene expression. It was essential that for any comparative gene expression study that the input RNA or cDNA from the test and control samples were equal.

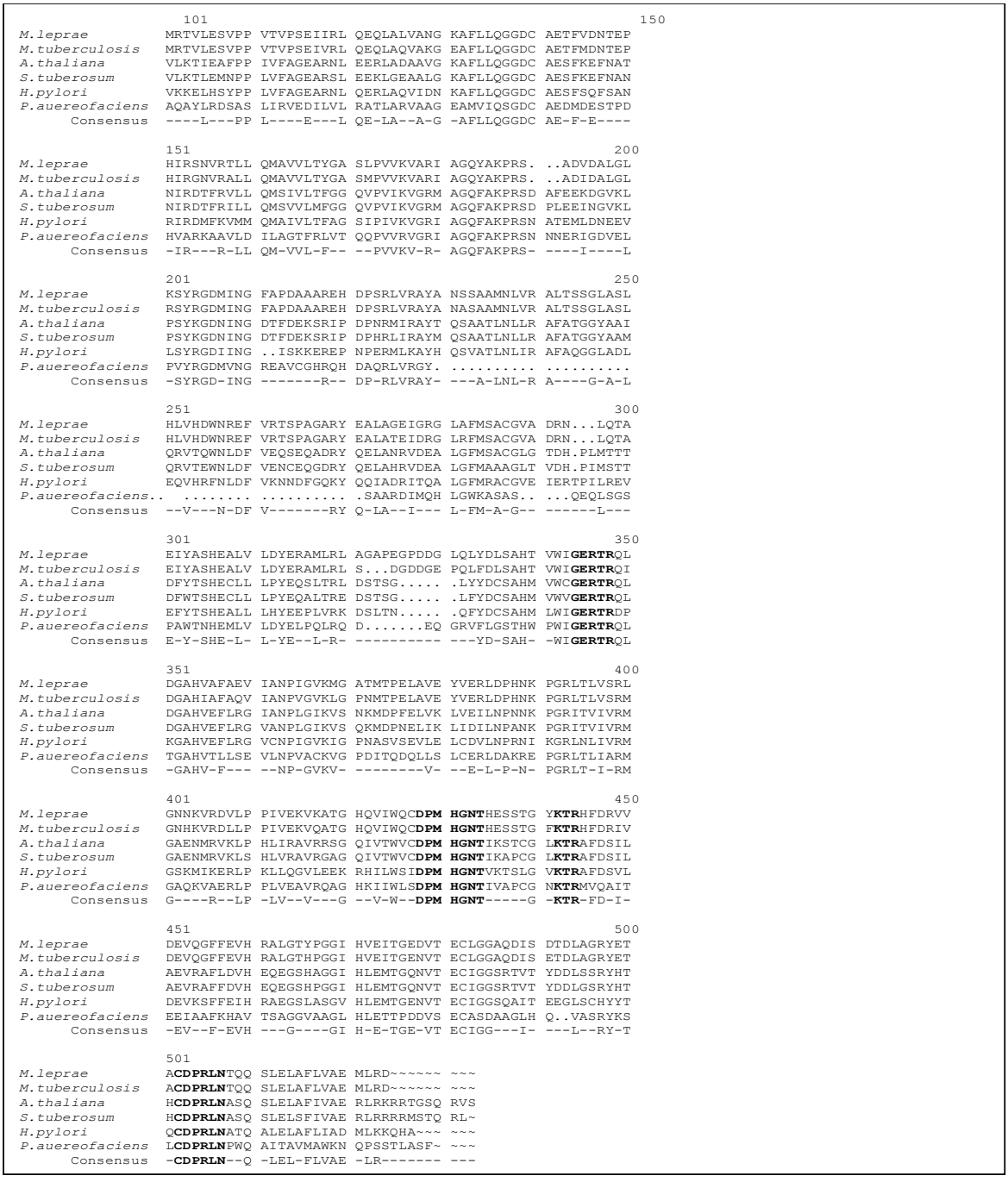

Figure 2. Alignment of the deduced amino acid sequence of the *M. tuberculosis* DAHP synthase with homologs from *Pseudomonas auereofaciens* *M. leprae* (AL022602), *M. tuberculosis* (AL021957), *Arabidopsis .thaliana* (M74819), *Solanum .tuberosum* (P37822), *H.pylori* (024947) and *P. auereofaciens* (L488339). The positions and identities of amino acids common to all the six proteins are in bold letters. The sequences for all DAHP synthase were obtained from GenBank at NCBI. For protein alignments, we used programs within the Genetics Computer Group sequencing Analysis Saftware package (GCG, Madison, USA).

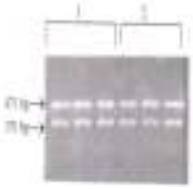

Figure 3 Competitive PCR for 16s cDNA to confirm equal proportion of cDNA used in the competitive PCR for *aroF* gene homolog competed in figure 4.

Figure 4. Competitive PCR for cDNA of *aroF* gene in intracellular bacilli and broth cultures of *M. tuberculosis*.Various amounts of *aroF* competitive DNA (2-fold dilutions of the competitive template) competed against a fixed concentration of *aroF* cDNA from intracellular bacilli and broth cultured *M. tuberculosis*.Lanes 1 - 4: Intracellular bacilli (in duplicate); lanes 5 - 8: broth cultured bacilli (in duplicate); lanes 9 & 10: PCR negative and positive controls, respectively and lane 11: 100 bp DNA ladder marker.

Determining the concentration of RNA in the test sample was particularly problematic owing to the contribution of the macrophage RNA despite the removal of host nucleic acids during selective lysis of the macrophages. Competitive RT-PCR was employed in order to rule out some background inherited using RNA dot blot hybridisation and the insensitive of the technique to detect small variation in the samples. Using this approach, putative *pepp* gene was found not to be upregulated using limiting dilution PCR and *htrA*-like gene was found also not to be upregulated after 15 hr of intracellular infection by quantitative competitive PCR (data not shown). There was at least 2 fold (2.3 ± 0.8) in the level of expression of *aroF gene* in *M. tuberculosis* as shown in figure 3 during intracellular infection of macrophages compared to the broth grown organism,

DISCUSSION

Our strategy in using synchronised method of infecting the macrophages was to force all the mycobacteria into the same stage in the infectious cycle with the harvesting of the RNA representing 15 hr of infection. The infection process was to favour the uptake of the bacilli by the macrophages in order to be able to detect the genes that are expressed in order to survive inside the macrophage on a long term basis i.e. the genes that are very important for the parasitic existence of the bacilli inside the macrophages which might include the metabolic genes. Differential lysis of the macrophages and M. tuberculosis (H37Rv) was adopted with the hope of removing majority of the macrophage nucleic acids.

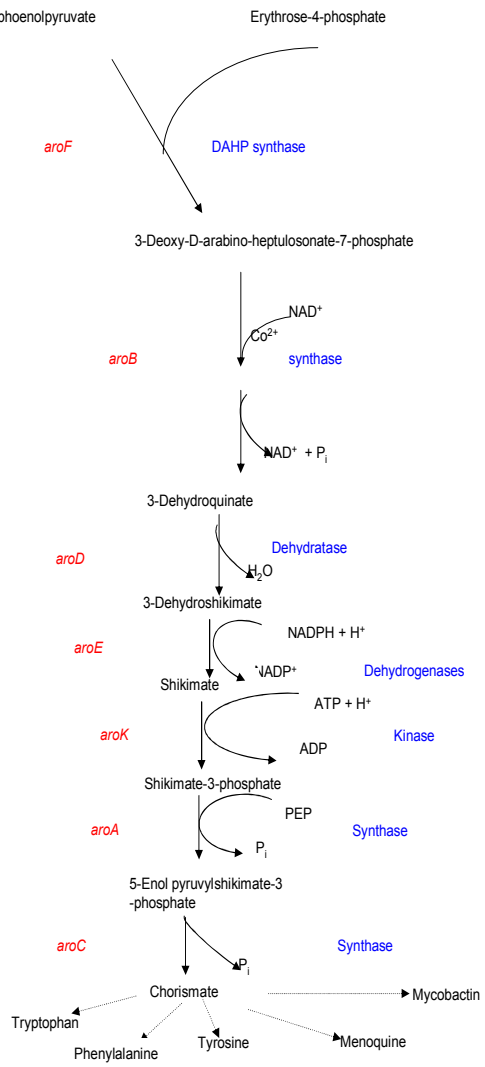

Figure 5: The pre-chorismate pathway of bacteria.

This method developed by Philip Butcher's group [16] exploited the differences in the cell architecture of the host and mycobacterium. The tender nature of the macrophage cell membrane makes it more susceptible to lysis than mycobacteria. This process of lysis has also taken into consideration of preserving the mycobacterial RNA with guanidine thiocyanate solution. Macrophage induced gene homologue has been found to be upregulated in M. tuberculosis based on the RNA extraction method [24]. With this strategy, the quantity of the RNA from mycobacteria within the intracellular environment could be roughly assessed for RAP experiment without a wide departure from the actual concentration. All together, three RAP products of interest were identified after eliminating the host genes and ribosomal genes for mycobacteria using 50 primer combinations. The low detection of differentially expressed gene might be as a result of using conventional PCR in amplifying cDNA which does not favour the amplification of DNA fragment with high G:C content. We are currently addressing this issue.

The *htrA-like* (serine protease) gene identified in this study was found not to be differentially expressed suggestive that this gene play little role in the intracellular survival of *M. tuberculosis*. This does not come as a surprise because of the presence of more than one related genes which could complement each other in functions. HtrA mutants of bacteria namely *Brucella abortus, Yersinia enterocolitica* showed decrease resistance to killing within macrophages [25, 26], but it is most likely that *htrA* mutant of *M. tuberculosis* will not behave in a similar way as with the other bacteria. Further characterisation of the *htrA-like* gene along with

the neighboring gene (putative two component regulatory gene) will throw some light on the pattern of expression and whether two-component regulatory gene does have some roles to play in controlling the expression of *htrA-like* gene in the early stage of infection. In addition to the genes identified in this study, we found upregulation of the *aroF* gene during intracellular infection of macrophages. The product of this gene is 3-deoxy-D-arabino-heptulosonic acid 7 -phosphate synthase (DAHP) which is the first enzyme in the shikimate pathwa with the final product of this pathway being chorismate - a precursor of many aromatic compounds (figure 5). The upregulation of *aroF* coding for this protein shows the importance of this particular pathway to the intracellular survival of *M. tuberculosis*. We could speculates that the increased in metabolic demand for the final product of this pathway in the synthesis of 2, 3-dihydroxybenzoic acid (a component of certain siderophores, which participate in the entry of iron into the cell) and p-hydrobenzoic acid (a precursor of the quinones) could lead to upregulation of *aroF* gene. For effective bacterial pathogenesis, the organism must sense its environment appropriately and respond with coordinate alterations in the expression of virulence genes. A number of environmental factors, including pH, osmolarity, temperature, and amino acid concentration, co-ordinately regulate the expression of virulence genes [27]. Since free iron is extremely limited in the mammalian host, a shift from a high to a low-iron environment is an important environmental signal to bacteria for coordinate regulation of gene expression. And to acquire this bound iron, bacteria have evolved a system for acquiring this element, in case of mycobacteria - a mycobactin and the precursors for the synthesis of this compound is the chorismate. We further speculate that in order to carry out this function i.e. iron acquisition from transferrin, it makes sense that the pathway leading to the synthesis of this compound is upregulated. Another condition that could lead to the upregulation of this gene is the differences in the oxygen tension between intracellular environment of the macrophage and broth culture environment. Although standing culture was used as the control but there would still be a subtle difference in the oxygen tension. In order to maximise the survival chance of *M. tuberculosis,* the menaquinone synthesis has to be increased of which the precursor is chorismate and this could directly or indirectly control the expression of *aroF* gene. *Arabidopsis thaliana aroF* gene has been shown to be upregulated in response to pathogenic attack to *Pseudomonas syringae* [28] which is as a result of the induction of phenylalanine ammonia lyase - the first enzyme in phenylpropanoid pathway and other enzymes specific to lignin synthesis and secondary metabolism in order to provide aromatic precursors for the synthesis of defensive secondary metabolites to contain bacterial proliferation. Analogy of this phenomenon could happen in *M. tuberculosis* in order to produce precursors for the synthesis of

defensive secondary metabolites which could be antibiotic in nature. The *aroF* gene was also found to be upregulated in *Escherichia coli* grown in glucose, acetate, and glycerol media [29]. Chorismate/shikimate pathway is very important for bacteria survival, this probably explain why most preventable or curative approaches have been geared towards this pathway. The shikimate pathway in *M. tuberculosis* has long attracted attention as the potential target of finding a new vaccine or chemotherapeutic agent [30, 31]. In micro-organisms and plants, chorismic acid is a central precursor for the biosynthesis of an array of biochemically important and structurally diverse aromatic compounds. These include folic acid, vitamin K, ubiquinone and the three aromatic acids - phenylalanine, tyrosine and tryptophan [32]. The enzymes of the aromatic biosynthetic pathway are attractive targets for inhibitors since this pathway is absent in mammals. 6-Fluoroshikimic acid has been shown to be converted *in vivo* by the pre-chorismate pathway enzymes to ultimately produce inhibition of p-aminobenzoic acid (PABA) synthesis, and thus folic acid production [33, 34]. Mutants defective in the pre-chorismate pathway have vaccine potential. Aromatic-dependent mutants of the pathogenic bacteria *Salmonella spp*. which shares similarity to *M. tuberculosis* in being intracellular pathogen [35, 36], *Bordetella pertusis* [37], *Yersinia enterocolitica* [38], *Bacillus anthracis* [39] and *Aeromonas salmonicida* [40] have been shown to be avirulent and stimulate protective immunity. Para amino-salicylic acid is one of the drugs used in the treatment of tuberculosis; the drug mode of action is on this pathway by mimicking the compound p-aminobenzoic acid and thereby leads into wrongful incorporation of this agent in the folate synthesis thereby thwarting nucleotide synthesis. This drug is also known to have some inhibitory effect at some stage in iron metabolism [41]. This study confirms the importance of pre-chorismate pathway to the survival of *M. tuberculosis* during intracellular infection of macrophages. Before meaningful intervention can take place, there is need for detail knowledge of the enzymes of this pathway. Searching the mycobacterial DNA database, it appears that there is only one copy of *aroF* gene, as it is in *Streptomyces coelicolor* [42] which in contrast to *Staphylococcus aureus* which has more than one copy. This indicates that the disruption of this gene could be lethal to the organism since there is no gene to complement the function of this gene. Also, means the control of the *aroF* gene may be complicated in this organism since the product of the compounds it catalyses is a precursor of many metabolites including secondary metabolites. The other genes of pre-chorismic pathway are in clusters with the exception of *aroE* gene that codes for shikimate-5-dehydrogenase on the cosmid MTCY159 on the Sanger's sequencing database. In this study, we show that *aroF* gene expression is upregulated in *M. tuberculosis* during macrophage infection. In addition we provide plausible reasons for the importance of this pathway to the survival of *M. tuberculosis*

and demonstrate the value of the RAP-PCR in detecting some of the genes that might play important or additional roles during intracellular infection. Work is under progress in characterising the condition inside the phagocytes which could be multifactorial, in promoting the expression of this gene.

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