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Research paper

Characterization of ML0314c of *Mycobacterium leprae* and deciphering its role in the immune response in leprosy patients

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ABSTRACT

Mycobacterium leprae has a reduced genome size due to the reductive evolution over a long period of time. Lipid metabolism plays an important role in the life cycle and pathogenesis of this bacterium. In comparison to 26 lip genes (Lip A-Z) of *M. tuberculosis*, *M. leprae* retained only three orthologs indicating their importance in its life cycle. ML0314c (LipU) is one of them. It is conserved throughout the mycobacterium species. Bioinformatics analysis showed the presence of an α/β hydrolase fold and 'GXSXG' characteristic of the esterases/lipases. The gene was expressed in *E. coli* and purified to homogeneity. It showed preference towards short chain esters with *p*NP-acetate as the preferred substrate. The enzyme showed optimal activity at 45 °C and pH 8.0. ML0314c protein was stable between temperatures ranging from 20 to 60 °C and pH 5.0–8.0, i.e., relatively acidic and neutral conditions. The active site residues predicted bioinformatically were confirmed to be Ser168, Glu267, and His297 by site directed mutagenesis. *E*-serine, DEPC and Tetrahydrolipstatin (THL) completely inhibited the activity of ML0314c. The protein was localized in cell wall and extracellular medium. Several antigenic epitopes were predicted in ML0314c. Protein elicited strong humoral immune response in leprosy patients, whereas, a reduced immune response was observed in the relapsed cases. No humoral response was observed in treatment completed patients. Overexpression of *ml0314c* in the surrogate host *M. smegmatis* showed marked difference in the colony morphology and growth rate. In conclusion, ML0314c is a secretary carboxyl esterase that could modulate the immune response in leprosy patients.

1. Introduction

Leprosy, a chronic infectious disease of poverty, was recognized since the ancient civilizations of China, Egypt and India. It mainly affects the peripheral nerves, skin, eyes, mucosa of the upper respiratory tract and leads to loss of sensation and muscle paralysis, resulting in clawed fingers (Lastória and de Abreu, 2014). Due to improvement in health facilities, leprosy is now mainly confined to third world countries but the social stigma attached with it remains a big concern. *M. leprae*, the causative agent of leprosy is a slow growing obligate parasite. Similar to *M. tuberculosis*, it resides inside the body for years in a dormant state before reactivating itself in the host with a weak immune system (Gengenbacher and Kaufmann, 2012). They remain undiagnosed during this non-replicating state. It is a broad spectrum disease with tuberculoid leprosy at one end of the spectrum and lepromatous leprosy at the other end (Ilhan et al., 2007). Globally, 175,554 cases have been reported in 2014 and 213,899 new cases were reported in 2016. A new global strategy was launched by WHO– "*The Global Leprosy Strategy 2016–2020: Accelerating towards a leprosy-free world*" which aims to create a leprosy free world and avoid disabilities, especially among children affected by the disease in endemic countries ("World Health Organization, Global Leprosy Strategy 2016-2020,", 2017).

M. leprae is a member of *M. tuberculosis* complex. It invades the peripheral nerves taking up shelter in Schwann cells in addition to macrophages. It survives in phagosomes and phagolysosomes of host macrophages (Armstrong and Hart, 1975). The entire survival strategy of this mycobacteria revolves around the various metabolic alterations that take place upon infection, during dormancy and upon activation (Hatzios and Bertozzi, 2011). Understanding the physiological role of various enzymes involved in the metabolic processes is a necessity to control the disease and find new drug targets.

Numerous reports have confirmed that lipids and lipid metabolism play a very important role in survival and pathogenicity of *M. leprae*

Abbreviations: OD, optical density; Mtb, *Mycobacterium tuberculosis*; PBS, phosphate buffer saline; WHO, World Health Organization; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt; NBT, Nitro blue tetrazolium; CD, Circular Dichroism

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(Kaur and Kaur, 2017). Lipids are present in several forms (cell wall components, lipid droplets, non-bilayer lipid arrangements etc.) and serve multiple roles from being a source of nutrition, providing rigidity, evading the host immune response to serving as virulence factors (Kaur and Kaur, 2017). *M. leprae* actively catabolizes fatty acids for energy (Williams et al., 2004). A recent study pointed to a central role for lipid droplets suggesting that lipid droplets recruitment represents a mechanism by which host-cell lipids are delivered to *M. leprae* in the phagosome which may further be used for deriving energy during the dormant state (Mattos et al., 2010). Foamy macrophage is a typical feature observed in mycobacterial infection as a result of lipid droplets (LD) formation (Russell et al., 2009). Lipolytic enzymes are more essential for mycobacterial survival than lipogenic enzymes. It has been reported that both *M. tuberculosis* and *M. leprae* utilize host lipids as main carbon source during dormancy (Wheeler, 2003). Although both seem to follow a similar survival mechanism inside the host, but the survival strategy of *M. leprae* involves regulation of host genes involved in lipid metabolism. There are 26 lip genes in *M. tuberculosis*, whereas, only three of these have their counterparts in *M. leprae*, including ML0314c (LipU), ML1899 (LipG) and ML0119c (LipE). Till date, not a single lipid degrading enzyme has been studied in detail from *M. leprae*. For the present study, we have chosen ML0314c for further characterization owing to the essentiality of its ortholog (Rv1076) in *M. tuberculosis* H37Rv for in vitro growth (Griffin et al., 2011). It is also conserved in all mycobacterium species. Moreover, LipU is a hormone sensitive lipase. A hormone sensitive lipase (LipY) in *M. tuberculosis* has been reported to be induced under starvation to utilize triacylglycerols (Deb et al., 2006).

2. Materials and methods

2.1. Strains, plasmids and growth media

E.coli strains DH5α with plasmids pET-28a (Novagene) and pVV16 were maintained in LB media with kanamycin (50 mg/ml) and shaking at 180 rpm. *E. coli* BL21 was used as host for pET-28a. pVV16 was a kind gift from Dr. S. Cannan, France. The *M. smegmatis* mc^2155 was grown in Middlebrook 7H9 media with appropriate shaking and kanamycin. *M. Leprae* genomic DNA was a kind gift from Dr. Mallika Lavania, Stanley Browne Laboratory, The Leprosy Mission, Shahdara, New Delhi, India.

2.2. Bioinformatics analysis

The gene sequence of *ml0314c* was retrieved from *M. leprae* database and BLASTp analysis was carried out at NCBI website. Protparam was used for analyzing various physical and chemical parameters including theoretical pI, molecular weight, extinction coefficient, aliphatic index and hydrophobicity index (Wilkins et al., 1999). The active site residues were predicted using alignment tool CLUSTALΩ (Sievers and Higgins, 2014). The 3D models of protein ML0314c was prepared by using Swiss Modeller (Schwede et al., 2003). The models were subsequently analyzed by using Pymol software. The epitope prediction was done using SVMTRip tool (Yao et al., 2012). The interactive network of ML0314c was predicted using STRING (Szklarczyk et al., 2011).

2.3. Cloning, expression and purification of ML0314c

The primers were designed by oligoanalyzer tool (IDT) for *ml0314c* for subsequent cloning into expression vector pET-28a. Owing to the very high GC content of the mycobacterium genome, the primers were designed using codon optimization approach without disturbing the amino acid composition of the protein ML0314c (Kumar and Kaur, 2014). The gene was amplified from the *M. leprae* genomic DNA using the primers Fwd-5′AGAATTCTTGGCCATCAGCGCCGGAG-3′ and Rev-5′GGAAGCTTCTAGAGGCTCTGCCTGTCTCTA-3′, with *EcoRI* and *HindIII* restriction sites, respectively. The amplified fragment was ligated with pGEMT vector (Promega) and used to transform *E. coli* DH5α cells. The positive clones were selected by blue white selection followed by colony PCR. The plasmid isolated from positive clone was further digested with *EcoRI* and *HindIII* and ligated with previously digested pET-28a vector with *EcoRI* and *HindIII*. The ligated product was used to transform *E. coli* BL21 DE3 cells. The positive clones were selected on the basis of colony PCR and slow plasmid migration. The cloned gene was sequenced.

The *E. coli* BL21 cells carrying recombinant plasmid pET-28a-*ml0314c* was initially cultured overnight at 37 °C in 5 ml liquid LB media containing relevant antibiotics in orbital shaker. Next day 1% of overnight grown culture was inoculated in a flask containing 200 ml of LB containing antibiotics. The conditions were optimized to express the recombinant protein in soluble fraction (J. Kaur et al., 2017). The culture was grown again at 37 °C in orbital shaker till OD had reached $A_{600} \approx 0.5$, i.e., log phase and finally induced with 0.5 mM IPTG for 3 h at 37 °C. After induction, cells were harvested and re-suspended in lysis buffer (5 ml for each 50 ml culture) followed by ultrasonication and suspension of the pellet in solubilisation buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The Ni-NTA column (Qiagen) was used for purification of rML0314c. The solubilised rML0314c was loaded onto the column (2 ml), washed with buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 6.3) and finally eluted with buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 4.7). The collected fractions were pooled and refolded in 0.5 M arginine by dropwise dilution of the protein. The protein was then dialyzed against 20 mM Tris-Cl buffer for a whole day and was concentrated to its original volume by using PEG 8000.

2.4. Substrate specificity

The substrate specificity of ML0314c protein was carried out using *p*NP ester of following chain length, *p*NP-acetate (C2), *p*NP-butyrate (C_4), *p*NP-octanoate (C_8), *p*NP-deconoate (C_{10}), *p*NP-laurate (C_{12}), *p*NP-myristate (C_{14}), *p*NP-palmitate (C_{16}), *p*NP-stearate (C_{18}) (Sigma Chemical Co. USA), dissolved as 10 mM stock in ethanol and used as 2 mM concentration. The enzyme activity was determined by slight modification of the method as described previously (Dosanjh and Kaur, 2002). To 750 µl of 50 mM NaH_2PO_4 (pH 8.0) 50 µl enzyme (appropriately diluted) and 100 µl of 2 mM substrate (*p*NP-butyrate) and 100 µl of 10 mM sodium deoxycholate was added. After 15 min, 0.25 ml of 0.1 M Na_2CO_3 was added in reaction mixture. The mixture was centrifuged at 10,000 × *g* for 10 min and the activity was determined by measuring absorbance at 420 nm in UV/Vis spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme, which liberates 1 µmol of *p*-nitrophenol from *p*NP-ester as substrate per min under standard assay conditions. The total enzyme activity was expressed in Units and specific activity was expressed as U/mg of protein.

2.5. Effect of temperature and pH on enzyme activity and stability

The effect of temperature on enzymatic activity was studied at different temperatures (20–80 °C) and pH 8.0. Thermo stability of the recombinant enzyme was carried out by pre-incubating the enzyme for 30 min (20–80 °C) followed by cooling on ice for 15 min, before the enzyme assay. The enzyme without incubation was taken as control (100%) and reaction mix without enzyme served as blank. The enzymatic activity was assayed at different pH (3.0–10.0) at optimum temperature. The enzyme stability at different pH was studied by pre-incubating 0.1 ml of enzyme with 0.1 ml of 50 mM buffer of different pH for 1 h at room temperature. The assay was carried out at optimum temperature and optimum pH with appropriate diluted enzyme according to the standard assay conditions. The enzyme without incubation was taken as control (100%).