

ABSTRACT

Tuberculosis is a deadly disease caused by bacteria of the genus *Mycobacterium*. One-third of the world's population is infected with *Mycobacterium tuberculosis*. Two million these deaths occur each year in immunocompromised AIDS patients. *M. tuberculosis* has co-evolved with humans for many thousands of years. The bacillus has developed tactics to overcome the immune defense system and multiply in the macrophage. At the interface of the host and pathogen interactions, there is an interchange of metals and electrolytes. The host on one hand reduces the availability of metals essential for pathogen survival, like manganese and iron, in the macrophage and increases potassium ions which reduce pH in the phagolysosome. The host also generates Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), to create toxic affects through interactions with metals and metalloproteins. *M. tuberculosis* copes with the hostile environment in the macrophage by preventing the acidification of the phagolysosome, secreting antioxidant enzymes such as alkylhydroperoxidase (AhpF) and peroxiredoxin (AhpC), superoxide dismutase, SodA and SodC, and catalase KatG through the SecA system. *M. tuberculosis* contains 28 metal transporters, among them there are 12 unique P-type ATPases. This is an unusually high number of P-type ATPases in an organism. These ATPases transport several monovalent and divalent metals (Cu^+ , Cu^{2+} , Ag^+ , Zn^{2+} , Na^+ , K^+ , Ca^{2+} , Cd^{2+} , Pb^{2+} , Mn^{2+} , Mg^{2+} , and Co^{2+}) across biological membranes, using energy from ATP hydrolysis. Our analysis has revealed that these P-type ATPases have homologs in other intracellular symbiotic/pathogenic bacteria and certain chemolithotrophic archaea and bacteria. A correlation can hence be drawn among these pumps and the capability of surviving in noxious environments and coping with adverse redox conditions. Possible substrates were identified by determining the consensus sequences in different helices of these ATPases. However, out of the 12 P-type

ATPases confirmed, transported substrate could be postulated for four of these proteins; CtpA, CtpB, CtpV and KdpB. Using bioinformatic approaches we have characterized the possible genetic environment of these genes. The transmembrane regions were analyzed for consensus sequences and the N-terminals and C-terminals were scrutinized for metal binding domains, and we were able to categorize these ATPases into P₁ type and P₂ type ATPases. In an attempt to determine the substrate specificity, two of these ATPases (CtpC and ctpG) were cloned and transformed into *Escherichia coli* cells. Cells expressing CtpC were grown in different concentrations of metals and pHs. In these experiments CtpC was found to show an interaction with copper and cadmium. Pure protein was obtained by His-tag purification and para-Nitro Phenol Phosphatase (pNPPase) assay was performed with different metals, it was found that copper and zinc activated the phosphatase activity of the enzyme; and cobalt and manganese were inhibitory. Inhibition of the pNPP assay could mean that there would be activation in the ATPase assay, meaning that cobalt and manganese could be possible substrates to this enzyme.

Keywords: P-type ATPases, Mycobacterium tuberculosis, CtpC, CtpG, macrophage.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. José M. Argüello for accepting me into his group. I would like to take this opportunity to thank my committee members Dr. José M. Argüello, Dr. Kristin K. Wobbe, Dr. Joseph B. Duffy, Dr. Reeta Prusty Rao and Dr. George A. Kaminski.

I would like to extend gratitude to Dr. Elif Eren and Patrick Arsenault for teaching me the basics in my first year.

Warm thanks to my lab mates Deli Hong, Jeanniffer Sabrina Guerrier (Jenny), Brad Kauffman, Hyungjoon Kim (Jooni), Xin Cheng and Nhu Le for sticking with me through the good and rough times in the turbulent research environment.

Special thanks to my husband, Sathya, for his unfaltering love and support through the experiences I have been through for the past couple of years. I cannot ask more from my mom, Ahalya Ananth (Uma), dad, Ananthakrishnan Kondhaswamy (Ananth) and grandfather, Venkatesan Purushothaman (Venky) for their strong support in anything I endeavor.

These special people deserve special mention for the good times I have shared with them: Ann Mondor, Dr. James P. Dittami, Shwetha Srivastava, Charu Jain, Harita Haridas, Paola A. Pinzon, Prachi Gupta, Jeff Swana, Christina Ernst, Edith Plada, Paula Moravek, Dr. Uma Kumar, Dr. Destin Heilman, Vicktor Kiryak, Marta Dabros, Pranoti Navare, Peter Driscoll, Eftim Milkani, Moqing Hu, Christopher Zoto, Dough White, Jack Ferraro and Mike Ferraro.

Last but not least, I thank all the previous members of the lab, especially Maria Jose Orofino, Atin Mandal and Ying Yang for staying in touch with me and being such fine friends.

TABLE OF CONTENTS

ABSTRACT	i
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF FIGURES	vii
LIST OF TABLES	viii
LIST OF ABBREVIATIONS	ix
1. INTRODUCTION	1
1.1 <i>Survival in the Macrophage.</i>	1
1.2 <i>ROS and RNS</i>	2
1.3 <i>P-type ATPases.</i>	4
1.4 <i>Transmembrane Metal Binding sites and Classification of P-type ATPases.</i>	7
1.5 <i>N and C metal binding domains.</i>	11
2. MATERIALS AND METHODS	14
2.1 <i>Identification of homologues of <u>Mycobacterium tuberculosis</u> ATPases.</i>	14
2.2 <i>Identification of transmembrane helices.</i>	14
2.3 <i>Identification of Metal binding conserved residues.</i>	14
2.4 <i>Cloning and Expression of ctpC and ctpG.</i>	15
2.5 <i>Growth curves.</i>	16
2.6 <i>Heavy metal inhibition assay.</i>	16
2.7 <i>Metal content in <u>E. coil</u> expressing ctpC and ctpG.</i>	17
2.8 <i>ctpC Protein Expression and Purification.</i>	17

2.9	<i>pNPPase Assay of ctpC.</i>	18
3.	RESULTS	19
3.1	<i>P-type ATPases in <u>Mycobacterium tuberculosis</u>.</i>	19
3.2	<i>Genomic organization of <u>Mycobacterium tuberculosis</u> ctps</i>	23
3.3	<i>Homologs of the Ctps identified in other organisms</i>	24
3.4	<i><u>Mycobacterium tuberculosis</u> has more P-type ATPases than most known organisms.</i>	26
3.5	<i>Metal binding conserved residues in the transmembrane helices were identified.</i>	27
3.6	<i>Analysis of the role of ctpC</i>	30
3.7	<i>Heavy metal inhibition assay showed decrease in inhibition zone for Cd, Ni and Cu.</i>	34
3.8	<i>ctpC and ctpG expressing <u>E. coil</u> show accumulation of Co for ctpG.</i>	34
3.9	<i>ctpC was cloned and expressed to produce pure protein.</i>	34
3.10	<i>pNPPase Assay showed substrate level binding of Mn and Co.</i>	36
4.	SUMMARY	37
5.	REFERENCES	40

LIST OF FIGURES

Figure numbers	Page No.
Fig. 1: Structure of the monomeric unit of human superoxide dismutase 2 (SOD2), showing the Zinc cofactor in the centre.	3
Fig. 2: A schematic representation of the membrane topology of a P-type ATPase.	5
Fig. 3: Catalytic Mechanism of P _{1B} -type ATPases.	6
Fig. 4: Overview of the P-type ATPase Family.	8
Fig. 5: Phylogenetic Tree of the P _{1B} -type ATPases.	10
Fig. 6: Dendrogram depicting the homology of the Ctps.	22
Fig. 7: Figure showing the genomic organization of the ctps.	24
Fig 8: Homology of ctpE, F, H and I to other P ₂ and P ₃ ATPases.	29
Fig 9: Differences observed in growth curves with Cu, Cd and Fe in BL21 cells.	31
Fig 10: Differences observed in growth curves with Cu and Cd with LMG194 Δ CopA and RW3110 Δ ZntA cells.	32
Fig 11: Heavy metal inhibition assay on 2x YT agar plates.	33
Fig 12: Heavy metal quotas of <i>E. coli</i> transformed with ctpC and ctpG.	34
Fig. 13: PCR Amplification of CtpC and CtpG.	35
Fig. 14: Expression of Pure protein for ctpC.	35
Fig. 15: pNPPase activity of ctpC with different metals	36

LIST OF TABLES

Table numbers	Page No.
Table 1: Cytoplasmic N-terminus Metal Binding Domains of P _{1B} -ATPases	11
Table 2: Cytoplasmic C-terminus Metal Binding Domains of P _{1B} -ATPases	12
Table 3: P-type ATPases in Mtb	21
Table 4: Homologous sequences found in other organisms.	25, 26
Table 5: Maximum number of P-type ATPases in other organisms.	27
Table 6: Conserved residues identified in each helix.	28
Table 7: Conserved residues in N and C terminal domains.	30

LIST OF ABBREVIATIONS

AhpC: Peroxiredoxin
AhpF: Alkylhydroperoxidase
APS: Ammonium persulfate
ATP: Adenosine Tri Phosphate
ATPases: Adenosine Tri Phosphatases
ATP-BD: ATP Binding Domain
BLAST: Basic Local Alignment Search Tool
CDF: Cation Diffusion Facilitator
cDNA: complementary DNA
CTP: Cation Transporting P-type ATPase
DNA: Deoxyribo Nucleic Acid
DTT: Dithiothreitol
IPTG: isopropyl β -D-thiogalactopyranoside
KatG: Catalase-Peroxidase
Mtb: *Mycobacterium tuberculosis*
OD₆₀₀: Optical Density at a λ of 600nm
pBLAST: protein BLAST
PCR: Polymerase Chain Reaction
pNPP: p-Nitro Phenyl Phosphate
ROS: Reactive Oxygen Species
RNS: Reactive Nitrogen Species
NCI: No consensus identified
NOS: Nitric Oxide Synthase
SOD: Super oxide dismutase
SR: Sarcoplasmic Reticulum
TM: TransMembrane segment
TM-MBD: TransMembrane - Metal Binding Domain
W NDP: Wilson's Disease Protein
ZIP: Zinc-regulated transporter, Iron-regulated transporter Protein

The more you care, the stronger you can be.

Jim Rohn

If your heart acquires strength, you will be able to remove blemishes from others without
thinking evil of them.

The weak can never forgive. Forgiveness is the attribute of the strong.

Mohandas Karamchand Gandhi

All too often arrogance accompanies strength, and we must never assume that justice is on the
side of the strong. The use of power must always be accompanied by moral choice.

Theodore Bikel