Original Article:

Characterization of inosine monophosphate dehydrogenase from *Staphylococcus aureus* ATCC12600 and its involvement in biofilm formation

S. Yeswanth,[1] C.H. Lalith Kumar,[1] V. Swarupa,[1] U.V. Prasad,[1] D. Vasu,[1] Y. Nanda Kumar,[3] L. Srikanth,[1] K. Venkatesh,[1] G. Sowjenya,[1] P. Santhosh Kumar,[1] D. Kavya,[1] Abhijit Chaudhury,[2] P.V.G.K. Sarma[1]

Departments of [1]Biotechnology, [2]Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati and Department of [3]Zoology, Sri Venkateswara University, Tirupati

ABSTRACT

Background: In *Staphylococcus aureus* purine metabolism plays a crucial role in the formation of biofilm which is a key pathogenic factor. The present study is aimed in the characterization of inosine monophosphate dehydrogenase (IMPDH) from *Staphylococcus aureus* ATCC 12600.

Methods: IMPDH gene was amplified using primers designed from IMPDH gene sequence of *S. aureus* reported in the database. Then polymerase chain reaction (PCR) product was cloned in the Sma I site of M13mp18 and expressed in *Escherichia coli* JM109. The recombinant IMPDH (rIMPDH) was overexpressed with 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG); Michaelis constant (K_m), maximum enzyme velocity (V_{max}) and catalytic constant (K_{cat}) of expressed IMPDH were determined.

Results: The enzyme kinetics of IMPDH grown under aerobic conditions showed a K_m of 43.71±1.56 µM, V_{max} of 0.247±0.84/µM/mg/min and K_{cat} of 2.74±0.015/min while in anaerobic conditions the kinetics showed K_m of 42.81±3.154/µM, V_{max} of 0.378±0.036 µM/mg/min and K_{cat} of 4.78±0.021/min, indicating elevated levels of IMPDH activity under anaerobic conditions. Three-folds increased activity in the presence of 1 mM adenosine triphosphate (ATP) correlated with biofilm formation. The kinetics of pure rIMPDH were close to the native IMPDH of *S. aureus* ATCC12600 and the enzyme showed single band in sodium dodecyl sulphate polyacrylamide gel electrophoresis with a molecular weight of 53 KDa.

Conclusions: Elevated activity of IMPDH was observed in *S. aureus* grown under anaerobic conditions and this was correlated with the biofilm formation indicating the linkage between purine metabolism and pathogenesis.

Key words: *Inosine monophosphate dehydrogenase, Biofilm, Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus is the aetiological cause for a wide range of human infections, including abscesses, septicaemia, arthritis and endocarditis. The increased prevalence of methicillin resistant (MRSA) and the emergence of community-acquired MRSA have prompted investigations into the pathogenicity of this species and research into the development of novel antimicrobial agents.[1-4]

Inosine monophosphate dehydrogenase (IMPDH; EC 1.1.1.205) catalyzes the rate-limiting step in the de novo biosynthesis of guanine nucleotides and has an essential role in providing the necessary precursors for deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) biosynthesis.[5] It is also a key enzyme in signal transduction pathways that mediate cellular differentiation and transformation.[6,7] IMPDH inhibitors, which cause the cessation of DNA synthesis, have been used in diverse therapeutic applications such as immunosuppression, arthritis and the treatment of parasitic disease for example drugs like cellcept1 and mizoribine.[8-10] Because increase in IMPDH activity is associated with cell proliferation, the enzyme is also a possible target for cancer chemotherapy.[11,12]

Sequence analysis of all known IMPDH enzymes supports a distinction between the bacterial and

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Corresponding author: Dr P.V.G.K.Sarma, Head, Department of Biotechnology, Sri Venkateswara Institute of Medical Sciences, Tirupati 517507, India. **e-mail:** sarmasvims@gmail.com

eukaryotic enzymes. A deep branching of the bacterial and eukaryotic forms of IMPDH is observed upon phylogenetic analysis of the relationships among the various IMPDH genes.[13,14] The analysis indicates a general functional conservation of amino acid residues and suggests unique amino acid sequence signature for these kingdoms. The phylogenetic differences between IMPDH enzymes reflect their kinetic differences and differential sensitivity to inhibitors. Enzymes from mammalian sources show distinctly lower values for the michaelis constant (Km) for nicotinamide adenine dinucleotide (NAD) than do those enzymes from bacteria.[15-17] In addition, mammalian IMPDH enzymes are several orders of magnitude more sensitive to inhibition by mycophenolic acid (MPA) than are bacterial IMPDH enzymes.[8,18,19] It has been observed that the biochemical and kinetic differences between bacterial and mammalian enzymes are a consequence of the variance of specific, identifiable amino acid residues. Identification of the critical residues or combination of residues is a prerequisite for the rational identification of agents that specifically target the bacterial enzyme.

IMPDH is one of the key enzyme for regulation of de novo biosynthesis of purine nucleotides.[20,21] IMPDH catalyzes the NAD-dependent conversion of inosine mono phosphate (IMP), which serves as a branch point between the adenine and guanine specific branches, to xanthosine mono phosphate (XMP) which is the rate limiting step in de novo guanine nucleotide biosynthesis.[22] Inhibition of IMPDH causes a reduction in the guanine nucleotide pool with subsequent interruption of DNA and RNA synthesis which results in cytotoxicity. The reduction in guanine nucleotides also compromises the ability of G-proteins to function as transducers of intra-cellular signals. IMPDH is also an important enzyme required for the colonization of *S. aureus* and is involved in generating energy (ATP) for increased cell wall biosynthesis, small colony variants (SCV) and increased biofilm formation (Figure 1) are the characteristic features of vancomycin resistant *S. aureus* (VRSA) strains.[23,24] Owing to the importance of IMPDH in *S. aureus*, the present study is aimed in the characterization of IMPDH of American type culture collection (ATCC) of *Staphylococcus aureus* 12600.

MATERIAL AND METHODS

In the present study, chemicals were obtained from Sisco Research Laboratories Pvt. Ltd., India;Hi-Media Laboratories Pvt., Ltd., India; Bangalore Genei Pvt., Ltd, India; Sigma-Aldrich, USA; New England Biolabs, USA; and QIAGEN Inc, Valencia, Netherlands.

Bacterial strains and conditions

Staphylococcus aureus ATCC12600 and *Escherichia coli* JM109 were obtained from Bangalore Genei Pvt. Ltd, Bengaluru, India. *S. aureus* was grown on modified Baird Parker medium at 37 ºC. After overnight incubation, a single black shiny colony with distinct zone was picked and inoculated overnight in brain heart infusion (BHI) and Luria bertani (LB) broths at 37 ºC with constant agitation at 120 revolutions per minute (rpm) in environmental orbital shaker.[24] *S. aureus* ATCC12600 culture thus grown was used to characterize IMPDH.

Biofilm assay

Biofilm units were determined by performing the assay in 96-well microtiter polystyrene plates. 200 µl of the culture grown in LB and BHI broths was applied into each well and plates were incubated at 37 °C for 24 hours. After incubation supernatant was removed and wells were washed with phosphate buffered saline (PBS) pH 7.5-8, air dried and stained with 0.4% crystal violet for 15 min (Gram-stain). After washing twice with distilled water, the wells were air dried and the absorbance recorded in a micro plate reader at 570 nm (Abiofilm). Similarly biofilm was estimated using cultures grown in LB and BHI broth without constant agitation was also determined and taken as Agrowth. The biofilm unit (BU) was determined using the formula:[25] BU=Abiofilm / Agrowth.

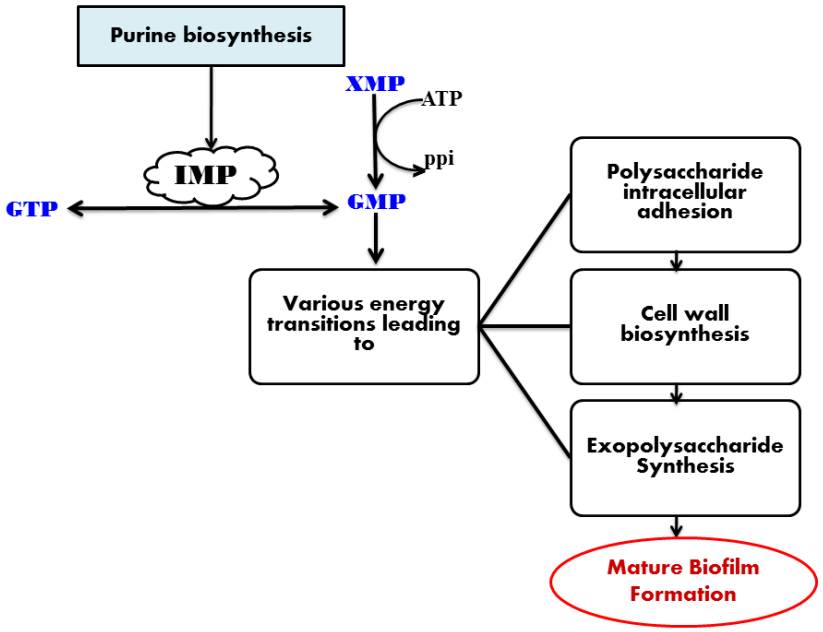

Figure 1: *de novo* purine biosynthesis leads to increased levels of GTP where XMP serves as precursor of GMP and subsequent increase in the levels of GMP through the action of digunylate cyclase and therefore higher levels of GMP results in increased biofilm formation by various energy transitions leading to polysaccharide intracellular adhesion, cell wall biosynthesis and exopoysaccharide synthesis

IMP=inosine monophosphate; ATP=adenosine triphosphate; GTP=guanosine triphosphate; GMP=guanosine monophosphate; XMP=xanthosine monophosphate; ppi=inorganic pyrophosphate

Enzyme kinetics of IMPDH

S. aureus ATCC12600 was grown in BHI at 37 ºC up to late log phase [optical density at 540 nm (OD_{540}=0.9)]. From the culture the cytosolic fraction was isolated[13] and used for enzyme kinetics of IMPDH. The 3 mL reaction mixture was prepared using 50 mM Tris HCl buffer (pH 8), 1 mM KCl, 1 mM dithiothreitol, 1 mM Ethylene diamine tetraacetic acid (EDTA), 0.05 mM NAD, 10 µM IMP and 50 µl of cytosolic fraction and incubated for 60 minutes at 37 ºC. The absorbance was measured at 340 nm against blank. Enzyme activity was expressed as concentration of product formed (NADH/minute/mL).[26-28] The maximum velocity of the enzyme catalyzed reaction was calculated by taking varying concentrations of substrate from 1 mM to 10mM and K_m and V_{max} was determined using Hanes-Woolf plot ([S] vs [S]/V) and sequences of IMPDH gene was searched from the Basic local alignment search tool (BLAST) data base to check the kinds of IMPDH in the *S. aureus*.

Amplification of IMPDH gene from *S. aureus* ATCC12600

Chromosomal DNA was extracted from late log phase culture of *S. aureus* ATCC12600 and IMPDH gene was amplified from chromosomal DNA using the primers forward 5'-CCCAGCACAATCTG-3'and reverse 5'-GAGTAGTTCGGTG-3', which were designed from sequence of *S. aureus* Mu50 strain. The reaction mixture consisted 500 ng of DNA, 50 pM of forward and reverse primers, 100 µl of deoxy ribonucleotide triphosphate (dNTPS) mix, reaction buffer that contained magnesium chloride ($MgCl_2$) supplied by the manufacture (Bangalore Genei Pvt ltd. India), 1 unit of hot start Taq DNA Polymerase (Bangalore Genei, Pvt., Ltd., Bengaluru India). The amplification parameters were calibrated in a Master Cycler Gradient thermocycler (Eppendorf; Germany) with an initial denaturation at 94 ºC for 10 min, followed by thirty five cycles of 94 ºC for 1 min of denaturation, annealing at 35 ºC for 40 s and amplifica-

tion at 72 ºC for 55 s. The cycles ended with final reaction at 72 ºC for 10 min. All the polymerase chain reaction (PCR) products were purified with nested primer PCR (NP-PCR) purification kit.

Cloning, expression and purification of IMPDH

IMPDH gene was cloned in the DNA restriction enzyme *Serratia marcescens 1* (Sma I) site of a phage vector derived from bacteriophage (M13) M13mp18 and expressed in *Escherichia coli* JM109 and the resultant clone was named as IMPDH1.[29] The insert in the clone was over expressed in 1mM isopropyl beta-D-1-thiogalactopyranoside (IPTG). The recombinant inosine monophosphate dehydrogenase (rIMPDH) was purified from the cytosolic fraction of IMPDH 1 clone. Presence of enzyme in the fractions was identified following the enzyme assay. In all the steps of protein purification the concentration of the protein was determined by Bradford's method.[31] The purity of IMPDH and its molecular weight was determined by performing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).[30]

RESULTS

In the present study the evaluation of biofilm units from *Staphylococcus aureus*ATCC12600 grown in both LB and BHI broths showed 0.081 BU and 0.092 BU respectively and indicates more biofilm formation in BHI broth. These high levels of biofilm formation were observed when *S. aureus* was grown in anaerobic conditions (Figure 2). The enzyme kinetics of IMPDH showed elevated activity in anaerobic conditions (Table 1). The enzyme kinetics of IMPDH in the cytosol, pure recombinant IMPDH and rIMPDH +1mM of ATP are shown in Table 2. The close K_m values of native IMPDH and rIMPDH corroborated with the sequence BLAST results indicating presence of only one kind of IMPDH in the *S. aureus* (Figures 3A, 3B and 3C). IMPDH gene that was amplified using PCR is shown in Figure 4A. The purity and molecular weight of uniduced cytosolic IMPDH , induced cytosolic IMPDH and rIMPDH establishing using 10% SDS PAGE are shown in Figure 4B.

DISCUSSION

Multidrug-resistant strains of *S. aureus* including strains resistant to vancomycin are increasingly being encountered all over the world. In such strains of *S. aureus*, conspicuous variations in morphology, physiology and growth characteristics have been observed due to high reductive conditions.[31-34] These conditions contribute in the upregulation of various genes involved in purine metabolism.[22] The central enzyme in purine metabolism is IMPDH which catalyses the formation of XMP which finally gets converted into GMP and which plays critical role in the energy transitions in the bacteria resulting in increased cell wall biosynthesis and biofilm formation (Figure 1). Studies of *Staphylococcus species*, which normally inhabit the human oral cavity, suggest that the key purine metabolic gene, purL in the purine metabolic pathway plays an important role in the oral biofilm formation known as dental plaque.[35-42] Further evidence is also available suggesting that purine is usually combined with sugars to participate in the synthesis of the polysaccharide components of cell walls and capsules causing biofilm

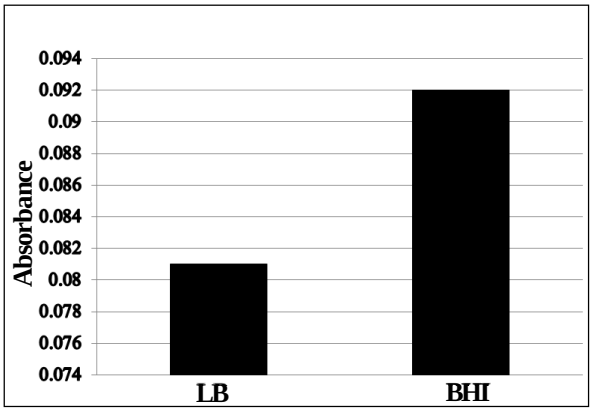

Figure 2: Evaluation of biofilm units in *Staphylococcus aureus* ATCC12600 grown in LB broth 0.081BU and in BHI broth 0.092 BU
LB=Luria Bertani; BHI=brain heart infusion; ATCC=American type culture collection; BU=biofilm unit.

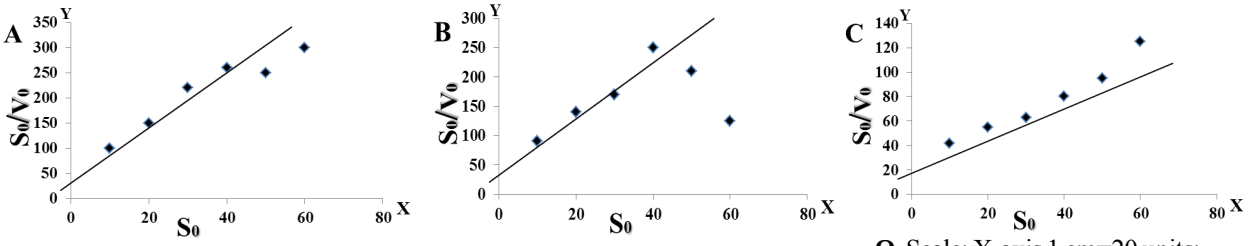

Figure 3: Kinetic plot of cytosolic fraction of IMPDH for the determination of K_m and V_{max} showing Hanes-Woolf Plot (A). Kinetic plot of rIMPDH for the determination of K_m and V_{max} showing Hanes-Woolf Plot (B). Kinetic plot of rIMPDH+1mM of ATP, for the determination of K_m and V_{max} showing Hanes-Woolf Plot (C)

K_m=Michaelis constant; V_{max}=maximum enzyme velocity; S_o=initial substrate concentration; V_o=initial velocity; rIMPDH=recombinant inosine monophosphate dehydrogenase; ATP=adenosine triphosphate

formation.[39] Therefore, it is reasonable to predict that biofilm formation is highly influenced by purine metabolism. In the present study *Staphylococcus aureus* ATCC12600 grown in anaerobic conditions showed elevated IMPDH activity and increased biofilm formation (Table 2 and Figure 3). The pure rIMPDH exhibited enzyme kinetics close to the native IMPDH indicating presence of only one kind of enzyme in *S. aureus* which also corroborates with sequence BLAST results among *S. aureus* strains (Table 2). The IMPDH enzyme showed 3 folds increased activity in the presence of ATP indicating the formation of XMP from inosine is probably due to the increased presence of phosphate donating groups thereby elevating the enzyme activity (Table 2). Thus, elevated purine metabolism contributes in the formation of SCV and biofilms which are the key pathogenic factors observed in both *S. aureus* infections and in episodes of relapse.

Table 1: IMPDH Kinetics of S. aureus ATCC12600 grown in aerobic and anaerobic conditions

Substrate	*S. aureus* grown in LB broth under					
	Aerobic conditions			Anaerobic conditions		
	K_m (µM)	V_{max} (µM/mg/min)	K_{cat}/min	K_m (µM)	V_{max} (µM/mg/min)	K_{cat}/min
IMPDH	43.71±1.56	0.247±0.84	2.74±0.015	42.81±3.154	0.378±0.036	4.78±0.021

Values expressed as mean ± SD from three determinants

LB=Luria Bertani; BHI=brain heart infusion; IMPDH = inosine monophosphate dehydrogenase; SD=standard deviation; ATCC=American type culture collection; K_m=Michaelis constant; V_{max}=maximum enzyme velocity; K_{cat}=catalytic constant

Table 2: Enzyme kinetics of IMPDH from *Staphylococcus aureus* ATCC12600

Source of IMPDH	K_m (µM)	V_{max} (µM/mg/min)	K_{cat}/min
Cytosolic fraction of *S. aureus* ATCC12600	42.81±3.154	0.378±0.036	4.78±0.021
rIMPDH	41.65±1.154	0.820±0.025	10.37±0.02
rIMPDH + 1 mM ATP	20.484±0.92	0.833±0.763	10.54±0.25

Values expressed as mean ± SD from three determinants

IMPDH=inosine monophosphate dehydrogenase;rIMPDH=recombinant inosine monophosphate dehydrogenase; ATP=adenosine triphosphate; SD=standard deviation; K_m=michaelis constant; V_{max}= maximum enzyme velocity; K_{cat}=catalytic constant

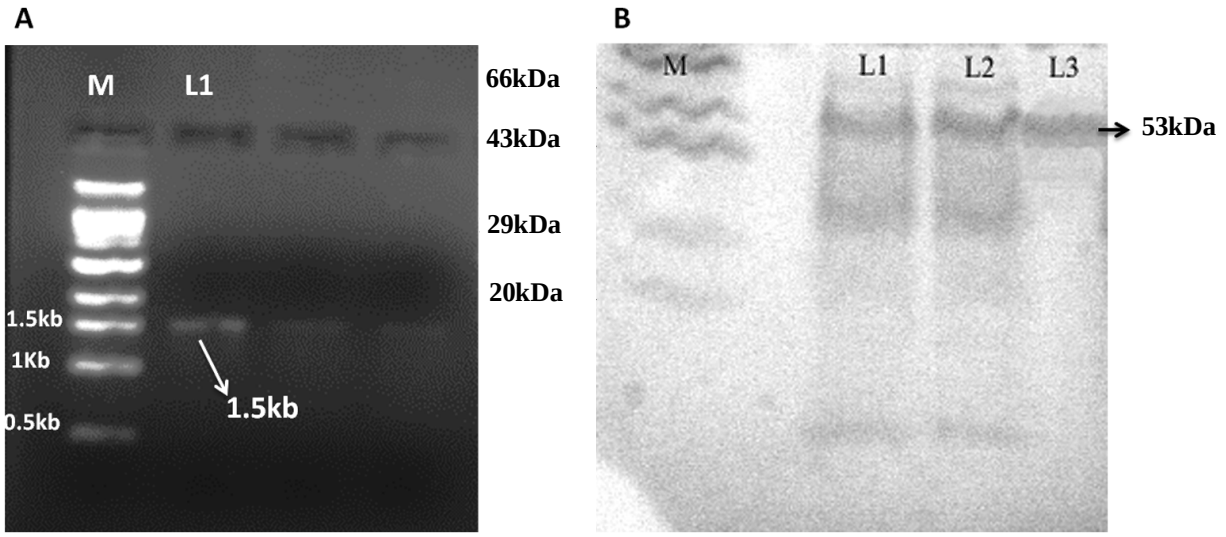

Figure 4: PCR amplification of 1.5kb IMPDH gene (L1) from the chromosomal DNA of *S. aureus* ATCC 12600 **(A)**. SDS-PAGE (10%) analysis of IMPDH. L1 = cytosolic fraction of uninduced IMPDH clone, L2 = induced cytosolic fraction of IMPDH1 clone, L3 = pure rIMPDH, lane M = molecular size marker obtained from Bangalore Genei Pvt. Ltd **(B)**

L = lane; PCR = polymerase chain reaction; IMPDH = inosine monophosphate dehydrogenase; SDS-PAGE = sodium dodecyl sulphate polyacrylamide gel electrophoresis; rIMPDH1 = recombinant inosine monophosphate dehydrogenase 1

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