Molecular characterization of *Nosema bombycis* methionine aminopeptidase 2 (*MetAP2*) gene and evaluation of anti-microsporidian activity of Fumagilin-B in silkworm *Bombyx mori*

Vijaya Gowri Esvaran¹ · Tania Gupta¹ · A. R. Narasimha Nayaka² · Vankadara Sivaprasad² · Kangayam M. Ponnuvel¹

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Abstract

Nosema bombycis is a spore-forming parasite causing microsporidiosis in silkworm *Bombyx mori*. Methionine aminopeptidase 2 (*MetAP2*), an essential gene of *N. bombycis*, is a target for the anti-microsporidian drug Fumagillin, an antibiotic derived from *Aspergillus fumigatus*. In this study, a 1077 bp full-length cDNA of the *MetAP2* gene of *N. bombycis* was cloned and characterized. Furthermore, the expression study of the *MetAP2* gene revealed a ubiquitous expression during all the developmental stages of the silkworm *B. mori*. The phylogenetic analysis of the *MetAP2* gene of *N. bombycis* revealed the *MetAP2* gene sequences to be highly conserved in nature. The present study also includes the validation of the anti-microsporidian drug Fumagillin against the *MetAP2* gene of *N. bombycis*. The findings revealed that Fumagilin-B could also suppress the *N. bombycis* multiplication in the silkworm *B. mori*, thereby proving the therapeutic role of Fumagillin against microsporidian infection. This is the first-ever report regarding the characterization of the *MetAP2* gene in the Indian isolate of *N. bombycis* and also towards the usage of Fumagillin in the control of microsporidiosis in *B. mori*.

Keywords Microsporidiosis · *MetAP2* · Fumagillin · *Nosema bombycis* · Anti-microsporidian

Introduction

In India, silk farming or sericulture is a labour-intensive and export-oriented agro-based industry. Silk production is severely hampered due to occurrence of various diseases caused by different microbial pathogens. Among them, pebrine is a highly mortal protozoan disease caused by *Nosema bombycis* (Microsporidia). Infection of this disease ranges from chronic to highly virulent nature. It is the only disease which comes from both primary/secondary infection and occurs during all the seasons. After the infection, silkworm larvae become inactive and slow in development

leading to death. Thus, there is an urgent need to develop or improve the available disease control methods to protect the silk industry from heavy loss.

Recent research works indicated that it is possible to control microsporidiosis using derivatives of benzimidazoles such as albendazole (Grob 2003) as they are active against many species of microsporidia. Fumagillin, ovalicin, and their analogues bestatin and TNP-470 have been demonstrated to have anti-microsporidian activity in in vitro and animal models of microsporidiosis. Fumagillin is an irreversible inhibitor of methionine aminopeptidase type 2 (*MetAP2*). The in vitro activity and effect of the inhibitors bestatin and TNP-470 on this recombinant microsporidian *MetAP2* was characterized (Zhang et al. 2005). These drugs do not bind or inhibit the activity of methionine aminopeptidase type 1 (*MetAP1*) or other aminopeptidases (Alvarado et al. 2009). Studies have demonstrated that Fumagillin derivatives display tissue and species selectivity in their ability to inhibit *MetAP2*, suggesting that it should be possible to design selective *MetAP2* inhibitors (Turk et al. 1998). The reactive epoxide of Fumagillin was found to form a covalent bond with histidine in the active site of human *MetAP2* (Liu et al. 1998). Application of Fumagillin to control nosemosis

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✉ Kangayam M. Ponnuvel
kmpvel@yahoo.com

¹ Genomics Division, Seribiotec Research Laboratory, Carmelaram-Post, Kodathi, Bangalore 560035, India

² Silkworm Pathology Division, Central Sericulture Training and Research Institute, Mysore 570008, India

in honey bees has been successful. The study provides a new tool for the development of in vitro systems to screen candidate compounds for use as new therapeutic agents for the treatment of microsporidiosis in silkworm. Hence, it was proposed to identify, clone, and characterize the therapeutic target *MetAP2* in *Bombyx mori* and study the effect of already reported anti-microsporidian drugs in controlling microsporidiosis in silkworm.

Materials and methods

Isolation of Nosema spores

A total of 100 bivoltine (CSR2) silkworm, *Bombyx mori* larvae infected with *Nosema bombycis* (NIK-5hm) at 0 day of fourth instar were utilized for the study. The *N. bombycis* spores were diluted to 10^8 spores/mL and fed to the silkworm. The silkworm larvae were reared till the emergence of moth and the spore was purified from the moth. The moths were macerated and ground using the mixer grinder and filtered through muslin cloth. The filtered spore suspension was washed 4–5 times with sterile-distilled water to remove the debris. Finally, the spores were laid over the percoll cushion and centrifuged at 10,000 rpm for 20 min. The pellet obtained contained the pure spores which were used for the extraction of DNA using a modified DNA extraction protocol (Reddy et al. 1999). The DNA extracted was dissolved in TE buffer, electrophoresed, quantified using Nanodrop, and used as a template for PCR analysis.

DNA isolation from infected tissue

Midgut tissues were dissected out at different hours of post-infection (hpi) in liquid nitrogen; genomic DNA was extracted using the modified shortcut DNA isolation protocol (modified at this laboratory). A small amount of tissue was excised from the silkworm and used for DNA extraction. The excised tissue or egg was placed in a microfuge tube and homogenized by addition of lysis buffer (0.1 M Tris cl, 0.01M EDTA, 1M Nacl) of about 500 μ L and crushed using

the micro pestle. The crushed tissue was briefly vortexed and centrifuged at 13,000 rpm for 5 min. The aqueous layer was transferred to a new microfuge tube, and an equal volume of freshly prepared phenol:chloroform:isoamyl alcohol (24:24:1) was added and the tube contents were mixed gently. The tube was centrifuged at 13,000 rpm for 5 min and the supernatant was transferred to a microfuge tube, and an equal volume of isopropanol was added and incubated at room temperature for 20 min followed by centrifugation at 13,000 rpm for 5 min to pellet the DNA. The pelleted DNA was washed with 70% ethanol and air dried. The dried pellet was dissolved in 50 μ L of sterile-distilled water and stored at -20°C for further use. The extracted genomic DNA was used as a template in PCR reaction with primers designed were used to amplify *MetAP2* gene.

Identification of MetAP2 gene

The detection of *MetAP2* gene through PCR technique has already been reported in *Nosema ceranae* (Higes et al. 2011). The *N. ceranae* *MetAP2* gene sequence (XM_00299649) was BLAST searched with *N. bombycis* genome (Microsporidia DB: <http://microsporidiadb.org>) and a sequence coding for *MetAP2* gene was retrieved from the database. The retrieved *MetAP2* sequence was used to design forward and reverse primers using Primer3 program. Around six different primers targeting the full length of *MetAP2* gene of *N. bombycis* were used for the study, and the position of the primers and the sequence were shown schematically in Fig. S1 and Table 1.

Cloning and characterization of MetAP2 gene

The presence of *MetAP2* gene in *N. bombycis*-infected samples was confirmed by cloning and sequencing of PCR fragments using pGEM T easy vector. The plasmid DNA was then purified through DNA purification column (Promega) and used for sequencing at Eurofins Genomics India Pvt. Ltd., Bangalore.

Table 1 Details of primers used for full-length cloning of *MetAP2* gene of *Nosema bombycis*

S. no.	Primer	Sequence (5'–3')	Primer-binding position	Product size (bp)
1	m1F	ATGAGGCCTATTGTTTATCAG	1–22	590
	m1R	GCTACTTACGCCGAATCAA	565–590	
2	m2F	CAAAGCTTTAGGTGTTGATGTAA	501–523	510
	m2R	GGATCGTATGTTGCACAATTT	994–1014	
3	m3F	CAGGTAAAGGTCGAATTTCTGAT	743–765	357
	m3R	TTACAAAAGGAGATGATTTTAA	1055–1077	

Multiple sequence alignment and phylogenetic analysis

The phylogenetic relationship of microsporidian *N. bombycis* *MetAP2* gene with *MetAP2* genes from other microsporidians was analyzed using the MEGA 6.1 (Kumar et al. 2004). The *N. bombycis* *MetAP2* gene was compared with the *MetAP2* amino sequences of the nine microsporidian species, namely, *N. apis* (JQ927010), *N. ceranae* (XM002996491), *N. bombi* (KF188775) *Encephalitozoon intestinalis* (AY224693), *E. romaleae* (JN039385), *E. cuniculi* (AF440270), and *E. hellem* (AY224694). The multiple sequence alignment was performed using clustal W program.

RNA isolation and cDNA synthesis

The RNA was extracted from different tissues midgut, trachea, Malpighian tubules, ovary, fat body, and egg using TRIzol reagent (Invitrogen, USA). The first-strand cDNA was synthesized using DNase free RNA sample (2 µg) along with 1 µL random hexamer (Eurofins India Pvt Ltd, Bangalore) and 10 mM dNTP (1 µL) was added followed by incubation at 65 °C for 5 min. Finally, 5X reverse transcriptase buffer (4 µL), 5 mM DTT (2 µL), and Superscript III reverse transcriptase (Invitrogen, USA) (1 µL) were added to obtain a final volume of 20 µL. The reaction mixture was incubated at 42 °C for 60 min and the reaction was terminated by heating at 75 °C for 10 min according to the manufacturer's protocol.

Tissue-specific expression profile

The expression of *MetAP2* in different tissues was analyzed using quantitative PCR (qPCR) with *MetAP2* gene-specific primer. One microliter of first-strand cDNA was used in a 20 µL reaction mixture using the specific primers exclusively designed for qPCR on STRATAGENE Mx 3005P real-time PCR system. The experiment was performed in triplicate. The results were standardized to the expression level of the constitutive β actin gene. A non-template control (NTC) sample was also run to detect contamination.

Selection of drugs and in vivo toxicity test

A wide range of drugs have been evaluated for efficacy against microsporidia under in vitro and in vivo conditions, and Fumagillin continues to be the most effective. Fumagillin-B is the only available drug to treat *Nosema* disease in North America (Williams et al. 2011) and its usage is allowed at special circumstances in Spain and a few parts of Europe (Stevanovic et al. 2013). The commercial products Fumagilin-B (Medivet Pharmaceuticals Ltd., Canada)

contain the therapeutic Fumagillin as the dicyclohexylammonium (DCH) salt at a concentration of 21 mg soluble Fumagillin base per gram. Fumagillin was successfully used to treat *N. apis* in honey bees. Hence, Fumagillin-B was evaluated for its toxicity at different concentrations 2, 20, 120, and 200 mg/mL orally to uninfected healthy larvae.

Evaluation of anti-microsporidian activity

About 500 silkworms were infected with *N. bombycis* spores at a concentration of 1×10^8 spores/mL, and among them, three trays containing 100 silkworms each were treated with Fumagillin-B (2, 20, 120 mg/mL). The samples were collected at about 24 h interval for 5 days. The RNA was isolated followed by cDNA synthesis which was used for further analysis. The multiplication of *N. bombycis* in the infected and Fumagillin-treated silkworm was quantified using the expression of *N. bombycis* specific genes. Along with the *MetAP2* gene, the polar tube protein and β-tubulin genes essential for *N. bombycis* infection were used for the study. A known concentration of tenfold serially diluted spore DNA samples was used as template to obtain the standard curve in an Agilent Stratagene Mx3005P qPCR instrument targeting the *Nosema*-specific β-tubulin gene. Standard curve was established by plotting the threshold cycle (Ct) on the Y-axis and the log of concentration on the X-axis, and is used to determine the concentration of the unknown samples. The spore load was determined by plotting the Ct value of infected and Fumagillin-treated silkworm genomic DNA samples.

Statistical analysis

The real-time expression data were subjected to descriptive statistical analysis and the significance of deference between infected and treated samples was tested using the analysis of variance, and *p* value of <0.01 is considered statistically significant.

Results and discussion

The examination of the purified *N. bombycis* spores isolated from the silkworms through the microscope revealed that their morphology was in confirmation with the earlier report (Selvakumar et al. 2005). Analysis of inoculated fourth instar larval samples revealed that the intensity of infection increased gradually as confirmed by microscopic examination and through the conventional PCR using the pathogen-specific primers. Methionine amino peptidases are metallo exopeptidases which catalyse the release of methionine from N-terminal amino acid of peptides and arylamides (Pandrea et al. 2005). *MetAP2* is the only known therapeutic target for

the anti-microsporidian drug Fumagillin. Hence, the full-length *MetAP2* gene of *N. bombycis* was cloned and characterized, and used for constructing phylogenetic tree for comparison with other microsporidians infecting the insects and humans. The drug Fumagillin was validated against the *MetAP2* gene of *N. bombycis* for controlling microsporidiosis in silkworms.

Cloning and characterization of MetAP2

This is the first report on the identification and characterization of methionine *MetAP2* gene in *N. bombycis*. The *MetAP2* gene primers were designed based on the *MetAP2* gene sequence (XM_00299649) of *N. ceranae*, a microsporidian-infecting European honey bees. As shown in Fig. S1, primers specific to the nucleotide sequence encoding for the *MetAP2* of *N. bombycis* were used to amplify the *MetAP2* gene. The amplicons with appropriate size confirmed the presence of *MetAP2* gene in *N. bombycis*. The amplified *N. bombycis MetAP2* gene fragments were cloned into TA cloning vector and the positive clones were sequenced. The sequence has been submitted to NCBI (KX820249). The ExPASy portal tool was used to confirm the open reading frame from the *MetAP2* nucleotide sequence coding for a large ORF of 358 amino acids. The estimated isoelectric point of *MetAP2* was found to be 5.75 and its molecular weight has been predicted to be 40.5 kDa. The major amino acids coded by *MetAP2* gene were isoleucine (10%), lysine (7.8%), and threonine (7.3%) with 47 negatively charged and 41 positively charged residues. The atomic composition of the *MetAP2* protein revealed it to be composed of 5691 atoms. The amino acids from number 59–354 were found to be conserved. The protein fold was found to contain both alpha helices and an anti-parallel beta sheet within two

structurally similar domains that are thought to be derived from ancient gene duplication. The blast X analysis of the *MetAP2* gene of *N. bombycis* revealed that no introns were present in the *N. bombycis MetAP2* gene.

Phylogenetic tree analysis

The cloned *MetAP2* sequence of the Indian isolate of *N. bombycis* was analyzed using Blastn and Blastp which confirmed that its DNA sequence had maximum homology with, *N. ceranae*, *N. bombi*, and *N. apis*. Neighborhood joining analysis revealed that the *MetAP2* gene formed three major clusters with highly significant bootstrap values. The first group comprised of *N. apis*, *N. ceranae*, *N. bombi*, and *N. bombycis*; the second included *E. intestinalis*, and *E. romaleae*, *E. hellem*, *E. cuniculi*, and *Ordospora colligata* formed an outer group (Fig. 1). Among the major clusters, the first group belonged to the human microsporidian pathogens, while the second group comprised of microsporidian-infecting insects. The second major cluster contains the three genotypes, namely, *Vittaforma*, *Spraguea*, and *Vavraia*.

Comparison of amino acid sequences of different microsporidians

The amino acid sequence alignment of nine microsporidian species (*N. bombycis*, *N. ceranae*, *N. apis*, *E. cuniculi*, *N. bombi*, *E. intestinalis*, *E. hellem*, and *E. romaleae*) is shown in Fig. 2. It is apparent from the sequence alignment that the nine microsporidian sequences are highly conserved between the amino acid segments of 66–112, 126–148, 155–168, 170–186, 201–214, 226–249, 288–299, 323–330, and 335–350, and they lie inside the predicted active site. The acidic NH₂-terminal domain of *MetAP2* coordinates

Fig. 1 Phylogenetic tree based on amino acid sequence of *Nosema bombycis MetAP2* gene compared with different microsporidian sequences retrieved from GenBank. The numbers near nodes indicate the number of bootstrap replicates; out of 100, bootstrap results greater than 95 are shown and the values lesser than 95 were removed. The scale indicates amino acid replacements per site

Fig. 2 Amino acid sequence alignment of *MetAP2* of eight different microsporidians; the conserved regions are shown in black boxes. The Fumagillin-B interacting amino acid residues at active site of

MetAP2 are indicated with black triangles and appear to be conserved completely in the microsporidian-infecting insects

two zinc ions and is involved in the activity of *MetAP2* responsible for the inhibition of phosphorylation of eIF2 by heme-regulated inhibitor kinase (Griffith et al. 1997). The *N. bombycis* lacks this polylysine sequence at the NH₂ terminal end, thereby indicating that it is not involved in the inhibition of phosphorylation of eukaryotic initiation factor. It has already been reported that the *MetAP1* gene lacks the carboxy terminal domain found between the active site Glu-243 and His-340 residues, thereby marking the specificity of Fumagillin towards *MetAP2* (Katinka et al. 2001). About 60% genetic distance was observed from the deduced amino acid sequence between *N. bombycis* and *Nosema* species-infecting insects (Table S1). The *N. bombycis* sequence was scavenged for conserved residues which play the critical role in binding of epoxide ring of Fumagillin. The amino acid residues Asp, Asp, Glu, His, and His were reported to be the highly conserved metal coordinating residues (Lowther et al. 1998). The conserved region indicates that the Fumagillin inhibits the enzyme in a similar way as reported for the

human enzyme (Molina et al. 2002). Even though a higher genetic distance was calculated, all the microsporidian isolates infecting insects and humans shared the most common conserved regions of the active site.

Tissue-specific expression profile

The qPCR analysis revealed high expression of *MetAP2* in the infected silkworm ovary followed by midgut, fat body, trachea, malpighian tubule, and egg where only slight expression was observed (Fig. 3). The upregulation of *MetAP2* gene in host ovary might be due to the stage at which the sample was collected, resulting in the transfer of the pathogen to its progeny via the egg. The mRNA transcript in egg might be due to the accumulation of *MetAP2* mRNA in the ovary. It has already been reported that the expression of *MetAP2* was upregulated immediately after infection and it is obvious that *MetAP2* should also have a higher expression to activate *MetAP2* gene of *Nosema*. To

Fig. 3 Expression level of *Nosema bombycis* *MetAP2* gene in different tissues of silkworm *B. mori* infected with *N. bombycis*

Fig. 4 *Nosema bombycis* *MetAP2* gene expression pattern in infected midgut tissues at different hours of post-infection (hpi). The β -actin gene is used as an internal control

further confirm the expression of *MetAP2*, specific analysis was carried out at different time intervals and the expression was found to be higher at 120 hpi followed by 72 hpi (Fig. 4). The *MetAP2* gene of *N. bombycis* was found to be expressed ubiquitously in all the developmental stages of the infected silkworm (Fig S2).

Effect of Fumagillin-B on *N. bombycis*

Earlier reports suggest that the Fumagillin binds to histidine in the active site of human *MetAP2* (Klein and Folkers 2003) and the carboxy terminal domain found between the active site Glu-243 and His-340 residues is absent in *MetAP1* (Katinka et al. 2001) marking its specificity towards *MetAP2*. The toxicity of Fumagillin reported in humans is due to the low selectivity of the drug between the microsporidian and human *MetAP2* (Van der Heever et al. 2014). Since microsporidia appear to lack *MetAP1* and carry only a gene for *MetAP2*, Fumagillin would be a promising drug for targeting only the microsporidian *MetAP2*. The toxicity is negligible in silkworms, since they do not show any sequence homology with *N. bombycis*. The absence of *MetAP1* would prevent microsporidia from compensating for the loss of *MetAP2* activity when exposed to Fumagillin. Earlier reports have already determined that Fumagillin inhibits only *MetAP2* protease activity of microsporidia and

does not interfere with the host genes (Didier et al. 2006). Phylogenetic analysis revealed that Fumagillin could be used as an anti-microsporidian drug against *N. bombycis* infecting silkworm *B. mori*. The efficacy of anti-microsporidian agent Fumagillin against *N. bombycis* was evaluated in our study. Bioassay analysis (cytotoxicity) was performed to confirm whether the selected concentration of compound Fumagillin is toxic or tolerable for the silkworms. Initially, four different concentrations of Fumagillin were selected, viz., 2, 20, 120, and 200 mg/mL per 100 larvae to study the anti-microsporidian activity. Each dose of Fumagillin-B was given orally to the healthy larvae on alternate days up to 120 hpi. The highest concentration of 200 mg/mL treated leaves was not consumed by the silkworms, whereas the silkworms treated with 120 mg/mL showed lethargic behavior. However, the silkworms treated with 2 and 20 mg/mL Fumagillin were at par with normal healthy silkworms without Fumagillin treatment, indicating that they did not produce any adverse effect on silkworm physiology. Hence, Fumagillin at three different concentrations were used to determine the effective dosage to control the *N. bombycis* infection in silkworms.

The efficacy of the drug Fumagillin in controlling the *N. bombycis* infection in the treated sample was compared with the untreated control through semi-quantitative and qPCR analysis. The results endorsed the effectiveness of Fumagillin in inhibiting the *MetAP2* gene of *N. bombycis* infecting silkworms. The Fumagillin was administered after 12 h of post-infection with *N. bombycis* spores. The *N. bombycis* multiplication was quantified using qPCR in Fumagillin-treated and control-infected samples. The *N. bombycis* multiplication was determined at 24 hpi interval up to 120 hpi targeting the *MetAP2* gene.

The effect of Fumagillin in *N. bombycis*-infected silkworms at five different time points by targeting *MetAP2* gene is shown in Fig. 5a. The expression level of *MetAP2* gene revealed increasing trend of multiplication up to 120 hpi. However, the Fumagillin treatment at different concentrations showed a significant decline in expression after 48 h after treatment, thereby indicating the suppression of *N. bombycis* multiplication. The treatment was given at three different doses 2, 20, and 120 mg/mL for 100 larvae. Among the three different doses, 20 mg/mL revealed a significant suppression of *N. bombycis* multiplication and the effect was consistently noticed at all time points. Though the treatment with the drug Fumagillin showed a significant decrease in target gene copies, it could not completely eliminate the pathogen from the host tissue. This finding indicates that Fumagillin treatment could significantly reduce the pathogen load but could not eliminate *N. bombycis* completely from the host tissue. Twenty mg/mL of Fumagillin-B was concluded to be the optimum dosage for controlling microsporidiosis with reduced symptoms and without affecting the silk production. The Fumagillin-treated silkworms were

Fig. 5 Effect of Fumagilin-B against the microsporidian, *Nosema bombycis* infection: the pathogen *N. bombycis* proliferation in silkworm *B. mori* was evaluated at 24 h interval up to 120 hpi. Fumagilin-B at three different concentrations (2, 20, and 120 mg/mL) was given and the experiment was performed under the laboratory conditions. The mRNA transcripts of pathogen genes were quantified using cDNA synthesized from total RNA of the infected and treated midgut tissues. The $p < 0.01$ are considered significant and are represented by asterisk. $p < 0.01^*$, $p < 0.001^{**}$, $p < 0.001^{***}$. **a** Effect of Fumagillin-B on MetAP2 gene expression of *N. bombycis*. **b** Effect of Fumagillin-B on β-tubulin gene expression of *N. bombycis*. **c** Effect of Fumagillin-B on PTP1 gene expression of *N. bombycis*

able to spin the cocoon, while the untreated batch failed to go for spinning. It was revealed that Fumagillin decreased the spore load drastically and delayed the spread of the disease. Even though Fumagillin inhibits *N. bombycis* to a greater extent by reducing the spore load inside the host, the drug administration should be done continuously to prevent the multiplication of the pathogen. The other two

essential genes β-tubulin and polar tube protein 1 involved in host invasion were also found to be expressed relatively at a lower level from 48 h after treatment when compared to the untreated control (Fig. 5b, c). The decline of spore load in the Fumagillin-treated sample was further confirmed by qPCR with the genomic DNA of the infected and treated silkworms using the β-tubulin gene of *Nosema bombycis* (Fig S3). Our study indicated that Fumagillin-B could not completely eliminate the *N. bombycis* from the host. However, it could significantly suppress the multiplication of pathogen in the infected silkworms. Furthermore, this problem can be overcome through systematic formulation of the dosage and by developing appropriate application schedules. Our study validated the *Nosema MetAP2* gene as a target for anti-microsporidian drug and will also help in designing new inhibitors against the microsporidians in near future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals.

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