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Expression, purification and functional characterization of AmiA of acetamidase operon of *Mycobacterium smegmatis*

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ABSTRACT

Regulation of gene expression is one of the mechanisms of virulence in pathogenic organisms. In this context, we would like to understand the gene regulation of acetamidase enzyme of *Mycobacterium smegmatis*, which is the first reported inducible enzyme in mycobacteria. The acetamidase is highly inducible and the expression of this enzyme is increased 100-fold when the substrate acetamide is added. The acetamidase structural gene (*amiE*) is found immediately downstream of three predicted open reading frames (ORFs). Three of these genes along with a divergently expressed ORF are predicted to form an operon and involved in the regulation of acetamidase enzyme. Here we report expression, purification and functional characterization of AmiA which is one of these predicted ORFs. Electrophoretic mobility shift assays showed that AmiA binds to the region between the *amiA* and *amiD* near the predicted promoter (P2). Over-expression of AmiA significantly lowered the expression of acetamidase compared to the wild type as demonstrated by qRT-PCR and SDS-PAGE. We conclude that AmiA binds near P2 promoter and acts as a repressor in the regulation of acetamidase operon. The described work is a further step forward toward broadening the knowledge on understanding of the complex gene regulatory mechanism of *Mycobacterium* sp.

1. Introduction

An understanding of the regulation of gene expression is fundamental to our comprehension of any infection process and can identify potential targets for disease prevention and therapy. A number of central gene regulatory functions govern the life and growth of pathogenic organisms, and these have been studied for decades. The acetamidase enzyme of *Mycobacterium smegmatis* is a highly inducible enzyme which enables the organism to utilize several amides, including acetamide and formamide, as sole carbon source (Draper, 1967). It is expressed at basal level in non-induced conditions or bacteria grown in nutrient rich medium. This enzyme can be induced 100-fold in the presence of simple chemical inducers like acetamide that results in increase of its protein concentration up to 10% of total proteins (Mahenthiralingam et al., 1993; Parish et al., 1997). The study of the expression of gene encoding this enzyme should provide a better understanding of how mycobacterial gene expression is regulated. Inducible gene expression systems that allows turning mycobacterial genes on and off during infection studies are powerful tools to studying the gene function and validation of drug targets.

Amidase is expressed by many bacteria including *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa* and fungi. Amidase operon of *P. aeruginosa* is well characterized and it shares similarity with the acetamidase operon of *M. smegmatis* (Wilson et al., 1996; Norman et al., 2000). Although there are similarities in the DNA/protein sequence and their function, the organization of genes and the regulation of amidase operon in *P. aeruginosa* is entirely different compared to acetamidase operon of *M. smegmatis*. In *M. smegmatis* the genes are organized as *amiADS* and *E* and the gene *amiC* is divergently transcribed (Parish et al., 1997; Narayanan et al., 2000). *P. aeruginosa* amidase operon has *amiEBCRS* genes in which *amiE*, *amiC* and *amiS* exhibit a percentage of sequence identity and significant structural similarity to their homologues in *M. smegmatis* operon (Parish et al., 1997).

The structural gene, acetamidase (*amiE*) along with 1.5 kb upstream to *amiE* region was sequenced and predicted to have three open reading frames (ORFs) named *amiA*, *amiD* and *amiS* (Mahenthiralingam et al., 1993). Parish et al., 1997 further sequenced 1.5 kb upstream to *amiA* and showed the presence of a divergently transcribed gene, *amiC* (Fig. 1). These predicted ORFs

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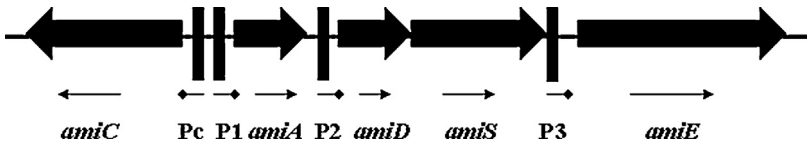

Fig. 1. Picture depicts the order of genes and positions of the promoters in the acetamidase operon. ORFs – are indicated by thick arrows and thin arrows depicting the direction of transcription. *amiA* negative regulator of amidase operon. *amiC* and *amiD* are proposed regulators. *amiS* is putative ABC transporter. *amiE* is the acetamidase gene. Promoter regions are indicated by bars with arrows indicating direction of transcription.

exhibit considerable homology with the regulatory and amide transporter proteins of other bacteria (Wilson and Drew, 1995; Chebrou et al., 1996). Previously, we had shown the presence of multiple transcripts of 3.0, 1.8 and 1.2 kb and mapped two transcriptional start sites at 1868 and 51 bp upstream to the start codon of acetamidase and hypothesized the involvement of either RNA processing or multiple promoters in the regulation of acetamidase operon (Narayanan et al., 2000). Subsequently, by promoter probe cloning and β-galactosidase assay, Parish et al., 2001 showed the presence of three promoters (Fig. 1). The first promoter is located between *amiC* and *amiA* (P1), the second is flanked by *amiA* and *amiD* (P2), which is amide inducible and the third one is present immediately upstream to *amiE* (P3). They have also identified a promoter oriented in opposite direction to other promoters, called Pc which expresses *amiC*. Studies with AmiA deletion mutants illustrated the negative role played by *amiA* and knockout of this gene resulted in amide independent, constitutive expression of acetamidase (Parish et al., 2001).

Further knockouts of other genes revealed the presence of multiple regulatory elements in acetamidase expression (Raghunand et al., 2006). By multiple gene knockout strategy, the authors showed that *amiC* and *amiD* act as positive regulators.

The DNA binding activities of these regulatory proteins are not studied before to identify the operator regions within this complex operon. Hence our lab was interested to study one of regulatory protein AmiA and its operator regions in the operon. In this study, we show for the first time the recombinant expression of AmiA and it's binding in P2 promoter region.

2. Materials and methods

2.1. Bacterial strains and media

Escherichia coli DH5α and *E. coli* BL21 (DE3) were obtained from Novagen. *E. coli* strains were grown in Luria Bertani (LB) liquid and solid (with 1.5% agar) medium at 37 °C. Kanamycin was supplemented at 40 μg/ml. *M. smegmatis* mc^2 155 was grown either in LB or Kohn–Haris minimal medium (NaCl 4 g, $MgSO_4$ 0.2 g, KH_2PO_4 2 g, $(NH_4)_3PO_4$ 2 g/l) containing 5 ml of trace elements ($ZnCl_2$ 40 mg, $Fe(NH_4)_2(SO_4)\cdot 6H_2O$ 200 mg, Cu_2SO_4 10 mg, $MnCl_2$ 10 mg, Borax 10 mg, $(NH_4)_2MoO_4$ 10 mg/l) with 0.05% Tween 80 at 37 °C with shaking (Kohn and Harris, 1941; Hopwood and Wright, 1978). Kanamycin was supplemented whenever needed at 20 μg/ml. Inducer (acetamide) was added whenever needed at appropriate concentrations as indicated elsewhere.

2.2. Plasmids and DNA manipulation

PCR product of 553 bp (coding region of *amiA*) was cloned using specific primers (Supplementary Table S1) with restriction enzyme site overhangs into the *BamHI-EcoRI* site of pET30(c) and in *E. coli* mycobacterial shuttle vector pMV261, and transformed into *E. coli* DH5α (Table 1). The resulting constructs were sequenced to confirm the fidelity of the sequence.

2.3. Expression and purification of AmiA

E. coli BL21 (DE3) harboring pETorf1 (*amiA* cloned into pET30(c)) was grown in LB medium containing 40 μg/ml of kanamycin at 37 °C to 0.6 OD at 600 nm and induced with 1 mM IPTG and continued to grow for further 2 h at 37 °C. The cells were placed in ice for 15 min, pelleted down by centrifugation at 6000 × *g* for 15 min and stored at −80 °C. The cells were thawed on ice, suspended in buffer A (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 10 mM imidazole, 20% glycerol, and 0.5 mM phenyl methyl sulfonyl fluoride) and sonicated on ice using SoniPrep 150 for 1 min with an interval of 5 min on ice for seven cycles. The lysate was centrifuged at 15000 × *g* for 15 min at 4 °C. The crude lysate was bound with ProBond Nickel affinity resin (Invitrogen) packed column by end-to-end shaking at 8 °C for 1 h. Column was washed with 60 column volumes of buffer B (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 40 mM imidazole, 20% glycerol, and 0.5 mM phenyl methyl sulfonyl fluoride) and recombinant AmiA was eluted with buffer C (50 mM NaH_2PO_4 pH 8.0, 300 mM NaCl, 200 mM imidazole, 20% glycerol, and 0.5 mM phenyl methyl sulfonyl fluoride). The resulting fractions were pooled and dialyzed against 1 l of 0.5× buffer A, concentrated on sucrose and stored at −80 °C.

2.4. Glutaraldehyde cross-linking of AmiA

Glutaraldehyde (1.5%) was added to AmiA protein in 0.5× buffer A to a final concentration of 0.05%, incubated at 37 °C for 15 min and cross-linking reaction was quenched immediately by addition of 6X SDS dye. The cross-linked products were analyzed in 12% SDS PAGE and visualized by Coomassie staining. The cross-linked products were subjected to western blotting and identified with anti-His antibody (Novagen).

Table 1
Plasmids constructed in this study.

Plasmid name	Description	Reference/source
pET30	*E. coli* expression vector, Kan^r, Lac promoter	Novagen
pCR2.1	TOPO cloning vector, Kan^r, Amp^r,	Invitrogen
pMV261	*E. coli* mycobacterial shuttle vector, Kan^r, *hsp60* promoter	Stover et al., 1991
pETOrf1	Coding region of AmiA in pET30(c)	In this study
pMVOrf1	Coding region of AmiA in *E. coli* Mycobacterial shuttle vector pMV261	In this study
pMVOrf2	Coding region of AmiD in *E. coli* Mycobacterial shuttle vector pMV261	In this study
pMVOrfC	Coding region of AmiC in *E. coli* Mycobacterial shuttle vector pMV261	In this study
pCML	1776–1975 bp fragment in pCR2.1 vector	In this study

2.5. Electrophoretic mobility shift assay (EMSA)

Primers were designed to amplify approximately 300 bp by designing overlapping primers for every 150 bp of the acetamidase operon (Supplementary Table S1) and labeled with [α^{32}P]dCTP using RediPrimeII random primer labeling kit (Amersham Pharmacia) as per kit protocol. Binding reaction was set-up with 1.2 ng of labeled DNA in 20 μl reaction mix containing 50 μg/ml double strand poly dI:dC, 50 μg/ml BSA, 1× binding buffer (25 mM HEPES, pH 7.5, 0.1 mM $ZnCl_2$, 1 mM DTT, 40 mM KCl and 5% glycerol) and 10% glycerol and incubated at room temperature (25–27 °C) for 1 h. This protein–DNA complex was resolved at 150 V in 0.25× Tris-borate-EDTA (TBE) for 4 h on 6% poly acrylamide gel cast with 2.5% glycerol in 0.5× Tris-acetate-EDTA (TAE). The gel was pre run at 100 V for 30 min; wells were thoroughly washed with buffer before loading the samples. After that run gel was dried at 80 °C for 10 min, exposed to photographic films and developed by autoradiography. EMSA experiments were also titrated with different DNA, protein and acetamide concentrations. Double-stranded oligonucleotides (ATTTCGGGTGAATGGAAAGGGGTGACCATG) and mutated oligonucleotides (ATTTCGGCCTGAATGGAAAGGGTTGACCATG) were prepared by mixing complementary single-stranded DNA oligomers in Tris-EDTA buffer, heating at 95 °C for 15 min and annealed by gradually cooling to 20 °C.

2.6. Foot printing analysis

The fragment which gave the band shifts in the EMSA, comprising 1776–1975 bp (Supplementary Table S1) of the operon was cloned into pCR2.1 vector using TOPO Cloning kit (Invitrogen). The plasmid was cut with *BamHI-XbaI*, the fragment was gel purified, and end filled by Klenow fragment, dephosphorylated with CIAP and then ends labeled with [γ-^{32}P] ATP by T4 PNK. Labeled fragment was cut with *XhoI* to ensure only one end was labeled. Probe was bound with 200 and 300 nM of purified AmiA using binding reaction conditions similar to EMSA. Protein DNA complex was treated with DNase I (0.15 U) as per kit protocol, phenol extracted, and ethanol precipitated and washed. Pellet was suspended in 8 μl of loading dye and 2 μl of 1 M urea, vortexed, heated to 95 °C for 2 min, snap cooled on ice and loaded on 8% sequencing gel (7 M urea, 40% formamide, 1× TBE) along with DNA sequencing ladder as per the kit protocol (USB PCR product sequencing kit).

2.7. In vivo over-expression studies

Wild type and recombinant *M. smegmatis* were transformed with the appropriate plasmids cultured in Luria Bertani Tween-80 (LBT) media supplemented with 20 μg/ml of kanamycin and were grown at 37 °C on shaking for 48 h. Cells were pelleted down at 4000 × *g* for 10 min, washed and suspended in Kohn-Harris medium with 20 μg/ml of kanamycin and grown at 37 °C overnight. Cells were exposed to heat shock for induction of *hsp*60 promoter at 45 °C for 2 h and continued to grow further at 37 °C for 3 h and then induced with various concentration of acetamide as indicated and allowed to grow at 37 °C. The culture was harvested by centrifugation at 6800 × *g* for 15 min and the pellet was preserved at −20 °C for further use.

2.8. RNA isolation and qRT-PCR

Total RNA was isolated from the over-expressed cultures using an RNeasy kit (QIAGEN, Inc.). The RNA was subsequently treated with DNaseI at 37 °C for 45 min. The DNase was then inactivated by incubation at 75 °C for 10 min. RNA was quantified by using a ND-1000 Nanodrop spectrophotometer (Nanodrop Technologies). Purified RNA was stored at −80 °C. For the determination of relative mRNA concentrations by quantitative reverse transcription-PCR (qRT-PCR), cDNA was synthesized with 2 μg of RNA using Superscript III first strand synthesis system with random hexamers (Invitrogen), according to the manufacturer's instructions. qRT-PCRs were carried out using Taqman PCR master mix (Applied Biosystems) according to manufacture's instruction using the Applied Biosystems 7300 real-time PCR system (Applied Biosystems). The qRT-PCR primers used in this study were listed in Table 2. To check for DNA contamination, control reactions for each sample were carried out in the absence of reverse transcriptase. The amplification conditions for all reactions were 1 cycle of 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

Table 2
Primers used for quantitative real-time PCR (qRT-PCR).

Primer name	Sequence (5′–3′)	Use
Ace F	ACCAACCCCATCTTCATGC	qRT-PCR
Ace R	TGTAGGCGTTGCGATTCTC	qRT-PCR
Ace Probe	AACGTGAGCCATTCGGAGTAGAGC	qRT-PCR
16s rRNA F	GGGAGCGAACAGGATTAGATAC	qRT-PCR
16s rRNA R	CCTTTGAGTTTTAGCCTTGCG	qRT-PCR
16s rRNA Probe	CGGTGGGTACTAGGTGTGGGTTTC	qRT-PCR
AmiA F	GGTCCGACTCTCACCAGAAC	qRT-PCR
AmiA R	CAGTCCGGTAGATGATCCCT	qRT-PCR
AmiA Probe	CGTCGATCGCCTTGTGTCGC	qRT-PCR
AmiD F	CCGACCTACACATTCCGTTG	qRT-PCR
AmiD R	GTGAATGTCGTCAGCCCTAC	qRT-PCR
AmiD Probe	TTCGATCTCACCTGCGCGATCTC	qRT-PCR
AmiC F	TAACAGGATTCGGCCATCG	qRT-PCR
AmiC R	CTCCACCGAAAACCTCTATGTC	qRT-PCR
AmiC Probe	CGTAGCTGCCCATCAGGTCCAT	qRT-PCR

The probes were labeled with 5′ FAM and 3′ TAMARA.

Analysis of qRT-PCR data were carried out using the comparative C_T method. For each qRT-PCR run, the calculated threshold cycle (C_T) was normalized to the C_T of the internal control 16S rRNA gene amplified from the corresponding sample. Statistical analysis was carried out using GraphPad Prism software. The data presented are averages of three independent experiments and error bars represent standard deviations.

2.9. SDS-PAGE and protein densitometry

Pellet generated from 30 ml of culture of over-expressed cells were lysed in 3 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 60 mM NaCl, 10% glycerol, 1 mM PMSF, 10 mM β-mercaptoethanol) by sonication for 5 min with an interval of 5 min on ice for a total of seven cycles. The cell-free lysate was collected by centrifugation at 15,000 × *g*, 4 °C for 15 min and stored in −80 °C. Protein concentration was determined by BCA kit (Pierce) as per the supplier's protocol. Cell-free lysates with equal protein concentration were resolved in 18.3 cm × 20 cm 10% SDS-PAGE at 30 V for 6 h, stained with Coomassie blue and gels (triplicate) were subjected to densitometer scanning and acetamidase concentration was determined by measuring the intensity of 47 kDa band using Quantity One software (Bio-Rad).

2.10. Estimation of amidase activity

To estimate intracellular amidase production, the acetamide induced cells were harvested and pelleted. The cells were resuspended in 20 mM PO_4 buffer (pH 7.4) and lysozyme was added to the cells (2 mg/ml) and stirred gently for 1 h at 4 °C. The cell-free lysates were collected by centrifugation at 20,000 × *g*, 4 °C for 20 min. This clear supernatant was used to estimate amidase activity after incubation with acetamide (100 mM). The intracellular acetamidase activity and release of ammonia was estimated by Nesselerization (Imada et al., 1973). All the experiments were

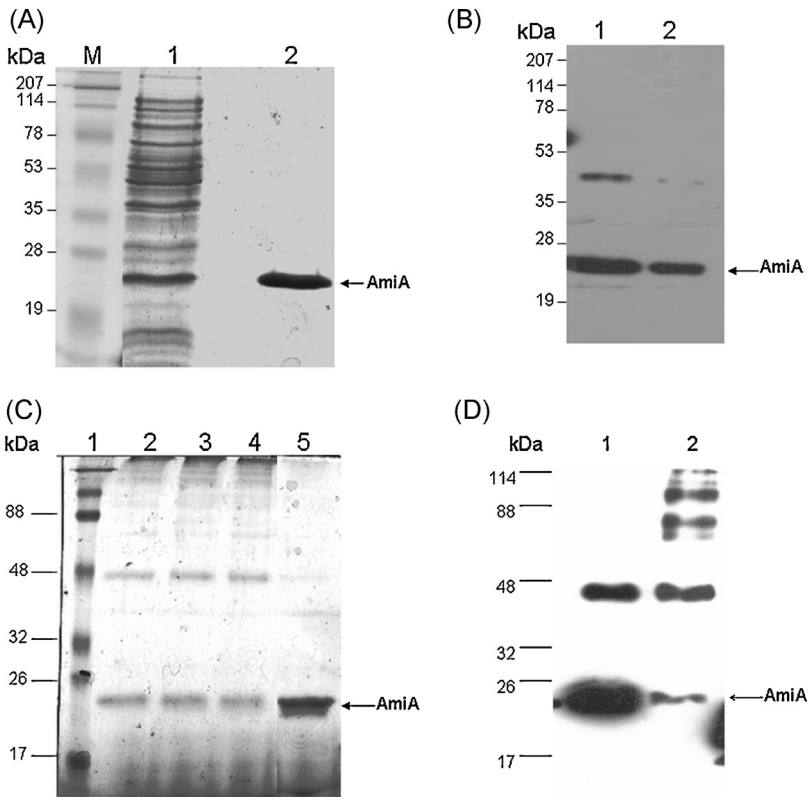

Fig. 2. Expression and characterization of recombinant AmiA. (A) Expression and purification of pETorf1.SDS-PAGE analysis of IPTG induced crude lysate of BL21 (DE3) cells carrying pETorf1 (lane 1) and Ni-NTA affinity chromatography purified AmiA (lane 2). M indicates molecular weight marker lane and Molecular weight standards were indicated in kDa. (B) Western blotting of crude lysate of *E. coli* BL21 (DE3) cells harboring pETorf1 (lane 1) and probed with anti-His antibody (Novagen). Molecular weight standards were indicated in kDa. (C) SDS-PAGE and Coomassie blue stained gel analysis indicating cross-linking of AmiA with 0.1% (lane 2), 0.01% (lane 3) and 0.05% (lane 4) of glutaraldehyde and control AmiA (lane 5). Molecular weight standards were loaded in lane 1. (D) AmiA (lane 1) was cross-linked by glutaraldehyde treatment (lane 2) as described in materials and methods and Western blotted using anti-His antibody.

performed in triplicate. One unit of acetamidase activity is expressed as amount of enzyme which catalyses formation of 1 μM of ammonia per minute under the assay conditions employed.

3. Results

3.1. Recombinant expression of AmiA

In order to determine the role of upstream genes of acetamidase expression, we constructed a vector to express one of the predicted ORF, AmiA. As shown in Fig. 2A, protein was expressed in *E. coli* system with predicted molecular weight of 24 kDa (AmiA), which was confirmed with same molecular weight band by Western blotting using anti-His (Novagen) antibody (Fig. 2B). Further recombinant AmiA was confirmed by mass spectrometry analysis (data not shown). AmiA is predicted to be part of MarR repressor family and expected to form oligomer. Glutaraldehyde cross-linking and Western blotting were performed to confirm the oligomerization of AmiA. As shown in Fig. 2C and D, glutaraldehyde cross-linking produced a range of bands (24, 48, 72, 96, 120 and 180 kDa). In SDS-PAGE, even without cross linking also we observed dimer band at 48 kDa when the protein was loaded in higher amounts (Fig. 2B and D, lane 1). Predominant band at 48 kDa indicates that AmiA forms dimer but possibility of higher order oligomer is not excluded.

3.2. AmiA specifically binds to the 1776–1975 bp fragment of P2 promoter

To detect interactions of the AmiA with the *cis* acting elements, EMSA was performed using the PCR generated [α-^{32}P] dCTP labeled DNA fragments. DNA binding ability of AmiA protein was screened throughout the operon using PCR generated fragments of ~300 bp length. Fragments of ~300 bp were generated using overlapping specific primers for every ~150 bp of operon in both directions (Supplementary Table S1). From the initial screening, it was established that AmiA specifically binds to fragment 1654–1975 and 1776–2104 bp of the operon. The binding was very specific and AmiA did not bind to fragments of 150 bp upstream and downstream to this sequence. The length of these fragments were further minimized to ~200 bp and found that the AmiA binds to fragment comprising 1776–1975 bp (Fig. 3A). Cold chase with increasing concentration of unlabeled probe (fragment 1776–1975) confirmed the binding specificity of AmiA with almost complete loss of the band at a 100-fold excess of competitor (Fig. 3B(I)). Cold chase with non-specific DNA (1173–1354 fragment) proved the specificity of binding of AmiA with fragment 1776–1975 (Fig. 3B(II)). Protein titrations were also performed using EMSA with a fixed DNA probe concentration (0.02 nM) over a range of AmiA concentrations (50, 100, 200, 300, 400 and 500 nM) (Fig. 3C).

Direct interaction between repressor and inducer was explored by EMSA with acetamide. No direct interaction was observed between repressor (AmiA) and inducer because the protein–DNA complex was not dissociated upon addition of acetamide (Fig. 3D). This result is contradictory to the MarR family of repressors that directly interacts with their inducers (Wilkinson and Grove, 2006). The fact that repressor–operator complex was not dissociated by addition of acetamide suggests that there is some other factor which senses acetamide levels inside the cell.

Foot printing analysis revealed that AmiA binds to a direct repeat (DR) sequence of GGGTGA spaced by eight bases (Supplementary

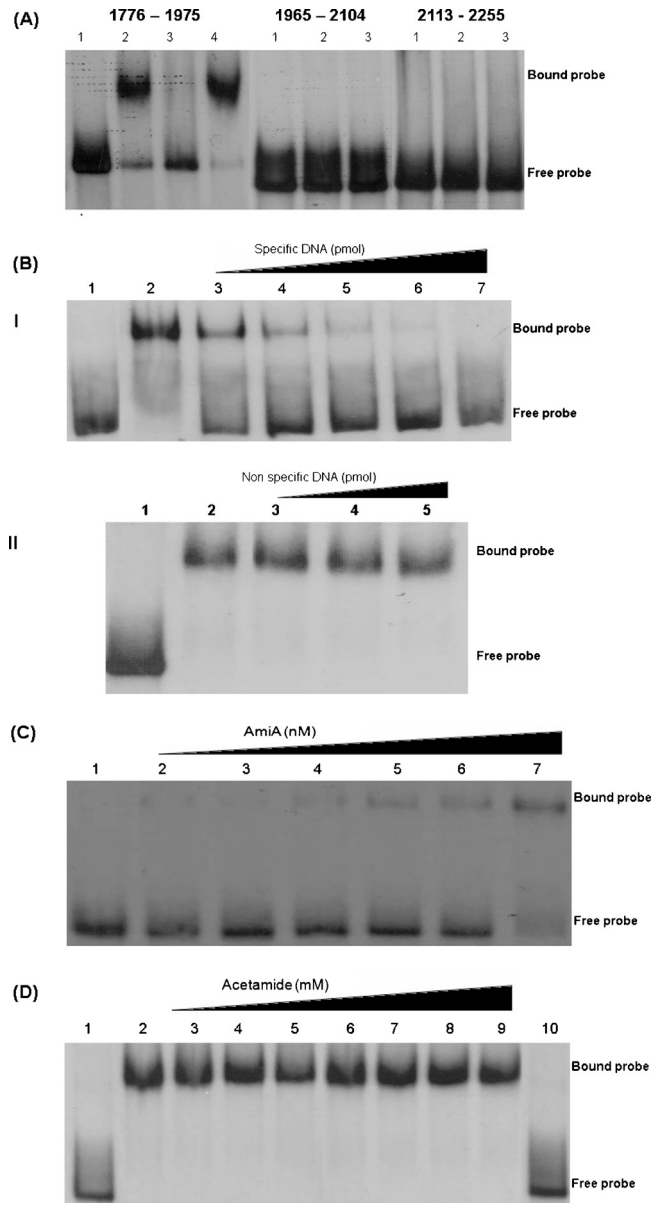

Fig. 3. EMSA analysis of AmiA binding. (A) EMSA analysis of AmiA binding to labeled probes. The probes (in bp) used are indicated above the lanes. Lane 1, labeled probe alone; lane 2, addition of labeled probe + AmiA; lane 3, addition of labeled probe + AmiA + unlabeled specific competitor (100-fold excess compared to labeled probe); lane 4, addition of labeled probe + AmiA + unlabeled nonspecific competitor (1173–1354 fragment was added 100-fold excess compared to labeled probe). (B) Cold chase experiment was performed for 1776–1975 probe with AmiA. Positions of free and bound probe are marked. (I) Lane 1, labeled probe alone; lane 2, addition of labeled probe + AmiA; lanes 3–7, labeled probe + AmiA with increasing amount of molar excess of unlabelled specific competitor (1776–1975) relative to labeled probe (20, 40, 60, 80, and 100-fold). (II) Cold chase experiment with non-specific competitor (1173–1354 fragment). Lane 1, labeled probe alone; lane 2, addition of labeled probe + AmiA; lanes 3–5, labeled probe + AmiA with increasing amount of molar excess of unlabelled nonspecific competitor (10, 50 and 100-fold). (C) EMSA was performed with increasing concentrations of AmiA with 1776–1975 fragment. Lane 1, labeled probe alone; lanes 2–7, labeled probe + AmiA titration with increasing concentrations of 50, 100, 200, 300, 400 and 500 nM. (D) EMSA was performed (1776–1975 + AmiA complex) with increasing concentration of acetamide. Lane 1, labeled probe alone; lane 2, addition of labeled probe + AmiA (500 nM); lanes 3–9, addition of increasing concentration of acetamide with labeled probe–AmiA complex (20, 30, 40, 50, 60, 70, and 80 mM); lane 10, labeled probe with acetamide (50 mM).

Fig. 1). DNA sequence analysis showed that this DR falls between the predicted transcription start site (TSS) and ribosomal binding site (RBS) and just two bases before the start codon of *amiD* gene, as outlined in Fig. 4A. A short, unlabeled double-stranded oligonucleotide containing this DR was able to compete with the labeled 1776–1975 bp DNA fragment for AmiA binding (Fig. 4B(I)). An otherwise identical oligonucleotide in which DR sequence was changed from GGGTGA to CCTTGA was not able to prevent the AmiA-mediated shift of the labeled oligonucleotide (Fig. 4B(II)). We propose that this six base pair DR sequence could act as an operator site for the acetamidase operon.

3.3. AmiA acts as a repressor

In order to study the influence of AmiA over-expression on acetamidase level, an AmiA over-expressing strain was created by cloning *amiA* gene under the control of *hsp*60 promoter in *Mycobacteria–E. coli* shuttle vector pMV261 (Stover et al., 1991). AmiA over expression was confirmed by qRT-PCR in induced and uninduced conditions at 0, 4 and 18 h time points (Fig. 5A). A significant increase in AmiA transcript level observed in over expressing strain as compared to uninduced cells at 4 and 18 h time points. We have used qRT-PCR to estimate the expression levels of acetamidase from AmiA over-expressing strain and its isogenic wild type strain. On addition of acetamide at increasing concentration (25 and 35 mM) there was a reduction in acetamidase transcript level in AmiA over-expressing strain at 4 and 18 h time points as compared to wild type strain (Fig. 5B and C). A significant decrease in acetamidase transcript at 4 and 18 h suggests that AmiA acts negatively on the transcription of acetamidase. These results were again confirmed by estimating acetamidase level in SDS-PAGE and densitometry analysis. We studied the acetamidase level on addition of different concentration of acetamide (inducer) at 18 h after induction. As observed by SDS-PAGE, wild type cells harboring no extra copy of AmiA, expressed high level of acetamidase. However, the cells which were over-expressing AmiA expressed significantly lower levels of acetamidase than the wild type as observed by protein band intensity at 47 kDa by densitometry analysis (Fig. 6A and B). Further acetamidase activity of wild type and AmiA over-expressing strain was performed based on the release of ammonia due to amide hydrolysis. The AmiA over-expressing strain shows significant reduction in acetamidase activity than the wild type strain (Fig. 6C).qRT-PCR study along with the EMSA experiment shows that AmiA acts as a repressor, by binding just downstream to the promoter (proposed operator) region and blocking the RNA polymerase from transcribing. Over-expression of AmiC and AmiD using *hsp* promoter were also tested. Neither AmiC nor AmiD over-expression had significant effect on the levels of acetamidase enzyme as observed in gel electrophoresis (Supplementary Figs. 2 and 3). We have also estimated the transcript levels of AmiD and AmiC in AmiA over expressing strain (Supplementary Fig. 4). We observed that the expression of AmiD and AmiC is minimal at 4 h and increased transcript level at 18 h. This may be due to constitutive expression of these genes.

4. Discussion

It is becoming more evident that gene regulatory mechanisms also play a role in the virulence mechanisms (Av-Gay and Everett, 2000; Manganelli et al., 2004; Ryndak et al., 2008; Zhang and Xie, 2011). Hence it is imperative to study the mycobacterial gene regulation in order to understand the gene expression machineries of mycobacteria. Acetamidase promoter of *M. smegmatis* has been used as an inducible system for expressing *M. tuberculosis* proteins for drug screening and vaccine studies. The gene regulation of the

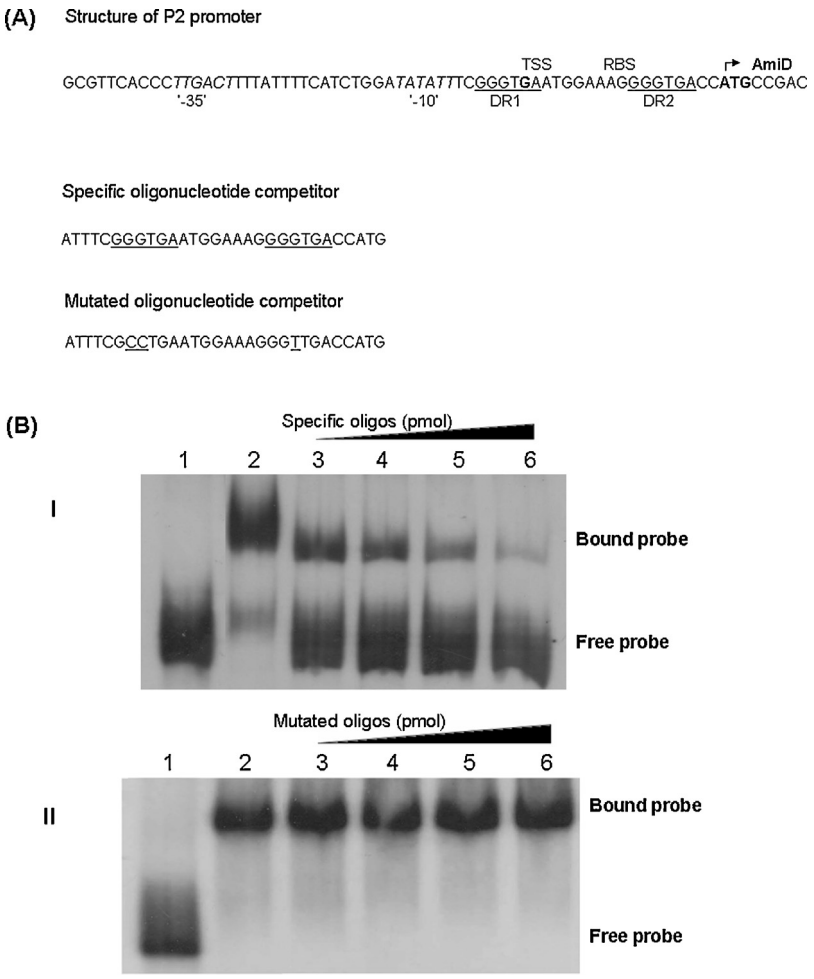

Fig. 4. (A) Sequences indicate the structure of P2 promoter. Italicized bases indicate the possible -35 and -10 boxes initially identified by Mahenthiralingam et al., 1993. Underlined bases indicate the direct repeat (DR) sequence with which the repressor AmiA binds and hinders RNA polymerase recognition. Transcription start site (TSS), ribosomal binding site (RBS) and the start codon of AmiD are indicated. Specific and mutated oligonucleotide sequences (underlined) used in the EMSA experiments are indicated. (B) Competition with oligonucleotide. (I) EMSA was performed (1776–1975 + AmiA complex) with increasing concentration of double-stranded oligonucleotide comprising DR sequences. Lane 1, labeled probe alone; lane 2, labeled probe with AmiA; lanes 3–6, double-stranded oligonucleotide titration with increasing concentrations of 50, 100, 200 and 300 pmol. (II) EMSA with mutated oligonucleotide. AmiA binding site in DR sequence has been changed (G ⟶ C, G ⟶ C and G ⟶ T). Lane 1, labeled probe alone; lane 2, labeled probe with AmiA; lanes 3–6, double-stranded mutated oligonucleotide titration with increasing concentrations of 100, 200, 300 and 400 pmol.

acetamidase gene which is the first inducible promoter of mycobacteria is complex. Hence we were interested to unravel the complex molecular regulation of the acetamidase operon of *M. smegmatis* step by step and to define the operator and promoter regions.

In this study we have shown the recombinant expression and purification of AmiA and defined the function of AmiA in the regulation of the operon for the first time. By knocking out the *amiA* gene and monitoring the acetamidase levels, Parish et al., 2001 demonstrated the negative role played by AmiA in the regulation of acetamidase operon. The amide inducible P2 promoter in the wild type was constitutively active in the *amiA* deleted strain. They had speculated that there might be some DNA regulatory elements functioning along with AmiA (Parish et al., 2001). We have earlier demonstrated that some proteins from the cell-free lysates of *M. smegmatis* grown with succinate binds to the OP2 near the P2 promoter but the identity of this protein was unknown (Subbian and Narayanan, 2007). Here, we have identified the protein to be AmiA and proved the repressor function of AmiA and characterized the operator region of acetamidase operon.

The reported operator is a hexanucleotide DR spaced by eight bases (GGGTGA-N8-GGGTGA) which exactly falls downstream to the predicted -10 box and extends from the TSS to RBS (Fig. 4A). This is contrary to the MarR family of repressors which always binds directly to palindromic sequences (Wilkinson and Grove, 2006). Only a few reports are available on binding of prokaryotic repressors to DR sequences. Lambda phage promoters controlling the lytic/lysogenic cycles of the phage (Ho et al., 1983), *chi-63* promoter of chitin catabolizing genes of *Streptomyces* sp. (Delic et al., 1992) and *galP1* promoter of galactose metabolism in *Streptomyces lividans* and *Streptomyces coelicolor* are regulated by DR sequences (Mattern et al., 1993). MarR family repressor can dissociate from their cognate operators upon induction with specific inducers like phenolic compounds, aromatics and detergents (Wilkinson and Grove, 2006). Although AmiA is found to have conserved residues in ligand binding site, the operator–repressor complex is not dissociated by the addition of acetamide, which is an inducer. We propose that some other proteins might sense the amide levels and act as co-inducer for the induction of P2 promoter.

According to previous observations that *amiC* knockout strain is non-inducible, *amiA* and *amiC* double knockout strain is highly constitutive (Roberts et al., 2003) and based on our observation that *amiC* over-expressing strain shows no significant effect on acetamidase levels in the presence of intact *amiA* indicate that AmiC is important for the induction. Our *in vivo* over-expression studies

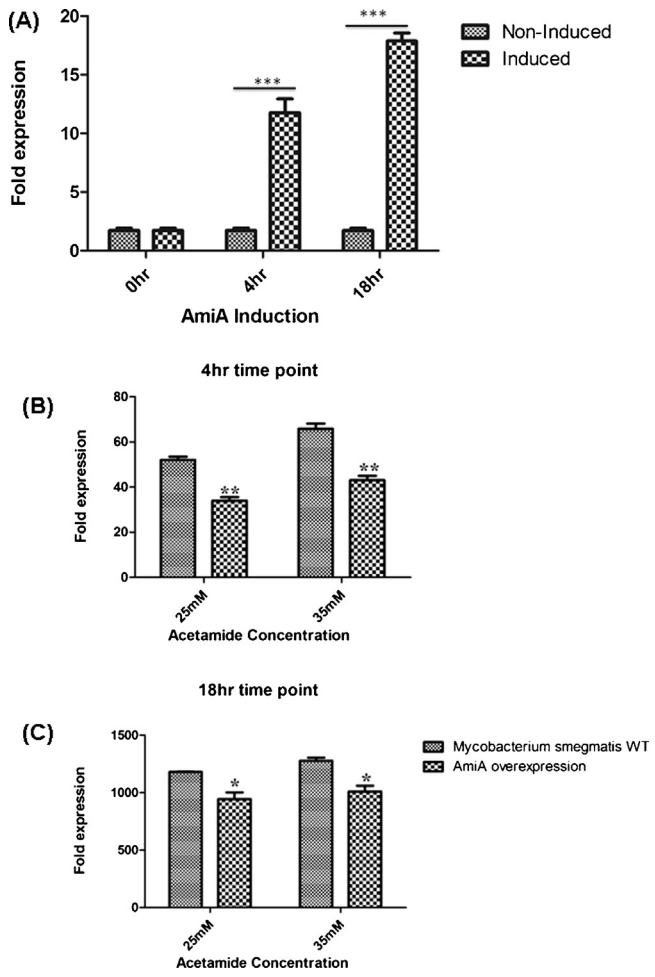

Fig. 5. qRT-PCR measurements of mRNA abundance in wild-type and *amiA* over-expression strains. Expression of *amiA* was quantified in *amiA* over-expression strains under induced and uninduced conditions (A). Relative mRNA abundance was determined for acetamidase (*amiE*) using total RNA at 4 h (B) and 18 h (C) after acetamide induction. The data represent average values and standard error measurements from three technical replicates, shown as percent relative abundance normalized to the *16s rRNA* gene. The symbols (*, **, ***) indicates $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

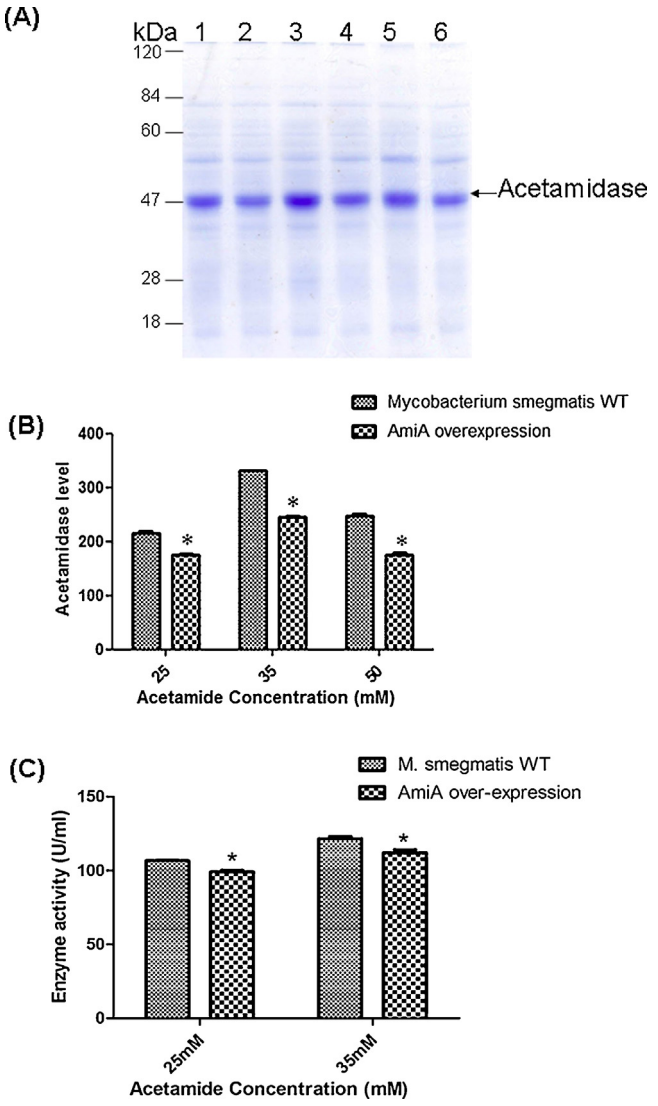

Fig. 6. Over-expression of AmiA and its effect on the expression of acetamidase. (A). *M. smegmatis* wild type cells and AmiA over-expressing cells were cultured and induced with acetamide for 18 h. Bacterial proteins were extracted and analyzed by SDS-PAGE. Lanes 1, 3 and 5 are crude extracts of wild type cells and lanes 2, 4 and 6 are crude extracts of AmiA over-expressing cells induced with 25, 35 and 50 mM acetamide respectively. Molecular weight marker is indicated by kDa. (B) Acetamidase level of wild type and AmiA over-expressed strains were measured using densitometer scanning and concentration was determined using Quantity One software in various acetamide induced concentrations (25, 35 and 50 mM). The symbol (*) indicates mean values that are significant ($P < 0.05$). (C) Acetamidase enzyme activity was estimated in wild type and AmiA over-expressed strains by estimating release of ammonia using Nessler's method at various acetamide concentrations. The symbol (*) indicates mean values that are significant ($P < 0.05$).

strongly demonstrated the physiological role played by AmiA as repressor. Thus, the characterization and identification of DNA binding sites of AmiA is an important step toward understanding the operon and will allow construction of more sophisticated expression system in future.

We thus refine the previous hypothesis of the regulatory mechanism of acetamidase operon. In uninduced conditions, constitutively expressed AmiA from P1 promoter blocks the expression of further downstream genes by binding near the P2 promoter. During induction, a small quantity of AmiC expressed by leaky expression of Pc may bind with amide and this complex in turn could bind with repressor AmiA and thereby could derepress the P2 promoter. Expression of P2 promoter ensures sufficient quantities of AmiD and AmiS. After sufficient quantities of AmiC, AmiD and AmiS have been produced, the expression levels P2 and Pc may be reduced and the newly formed AmiC and AmiD might positively regulate the P3 promoter (which is weak and constitutive in normal conditions). This in turn might increase the expression of acetamidase, AmiE to a large extent. Further work to investigate DNA binding activity of positive regulators and protein interactions between the regulators will provide complete understanding of the complex operon.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2014.02.011.

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