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Research article

amiA is a negative regulator of acetamidase expression in *Mycobacterium smegmatis*

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Abstract

Background: The acetamidase of *Mycobacterium smegmatis* is a highly inducible enzyme. Expression of this enzyme is increased 100-fold when the substrate acetamide is present. The acetamidase gene is found immediately downstream of three open reading frames. Two of these are proposed to be involved in regulation.

Results: We constructed a deletion mutant in one of the upstream ORFs (*amiA*). This mutant (Mad1) showed a constitutively high level of acetamidase expression. We identified four promoters in the upstream region using a β-galactosidase reporter gene. One of these (P_2) was inducible in the wild-type, but was constitutively active in Mad1.

Conclusions: These results demonstrate that *amiA* encodes a negative regulatory protein which interacts with P_2. Since *amiA* has homology to DNA-binding proteins, it is likely that it exerts the regulatory effect by binding to the promoter to prevent transcription.

Background

The acetamidase enzyme of *Mycobacterium smegmatis* is a highly inducible enzyme [1]. In the presence of a suitable substrate, such as acetamide, the enzyme is expressed to a high level, forming up to 10% of the total protein. In the absence of an inducer the enzyme is expressed at a low (basal) level. Previous work has shown that both positive and negative control elements are involved in the regulation of the acetamidase. Induction occurs at the transcriptional level with increased amounts of acetamidase mRNA detectable after 1 hour [2]. Three open reading frames have been identified immediately upstream of the acetamidase gene (*amiE* in Figure 1) and these have been proposed to encode two regulatory genes (*amiA* and *amiD*) and a transport gene (*amiS*) [2,3]. In addition, a third gene (*amiC*) which is divergently transcribed has been proposed to be a regulatory protein [3] (Figure 1).

The lack of a suitable highly inducible promoter has hindered research in the genetics of mycobacteria. The acetamidase promoter is a good candidate for use in such studies, but it is important to understand its regulation in order to improve its utility.

The aim of this study was to determine what role one of the proposed regulatory genes, *amiA*, plays in the regulation of acetamidase expression. An unmarked deletion mutant was constructed which lacked the *amiA* gene and the effect on acetamidase expression was assessed. We

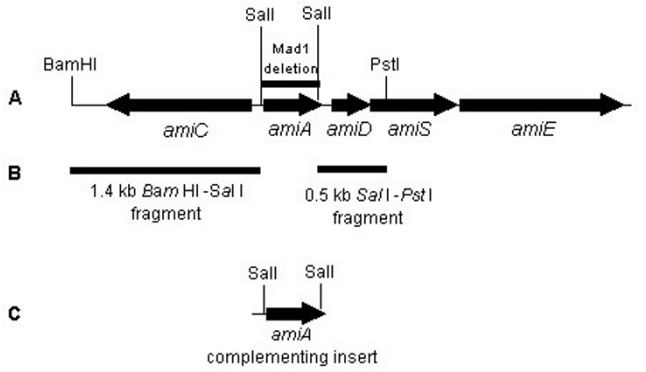

Figure 1
Vector constructs. **A:** Arrangement of genes in the *Mycobacterium smegmatis* chromosome. *amiA*, *amiC* and *amiD* are proposed regulators, *amiS* encodes one component of a putative ABC transporter. *amiE* is the acetamidase gene. **B:** The two fragments used to generate the delivery vector for construction of the *amiA* deletion mutant. The resulting *SalI* deletion in the Mad1 strain is indicated which removes the majority of the *amiA* gene. **C:** The region used to complement the Mad1 mutant.

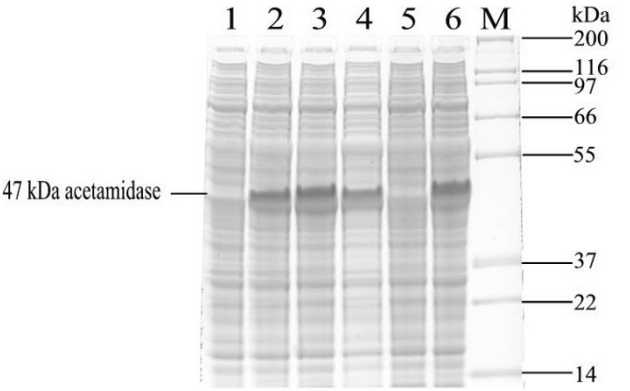

Figure 2
Acetamidase expression in Mad1. Cell-free extracts were electrophoresed though a 10 % acrylamide gel. Lanes are 1: wild-type MM-S 2: Wild-type MM-AS 3: Mad1 MM-S 4: Mad1 MM-AS 5: Mad1/pAGAN303 MM-S 6: Mad1/pAGAN303 MM-AS 7: markers, size given in kDa. The acetamidase band at 47 kDa is indicated. MM-S: minimal media with succinate. MM-AS: minimal media with acetamide and succinate.

also aimed to further define the promoter(s) responsible for acetamidase expression. Previous work with a reporter gene identified an inducible promoter within the region located approximately 1.5 kb upstream of the acetamidase itself [3], although the possibility of more than one promoter was not excluded. We used a β-galactosidase reporter gene to assay for promoter activity in the regions found immediately upstream of each gene. Four promoters were identified and their activity was measured in both the wild-type and *amiA* mutant strains.

Results and Discussion

Mad1 shows constitutively high level expression of the acetamidase

In order to begin to determine the role of the upstream genes in acetamidase expression, we constructed an unmarked *amiAΔ* mutant strain and then assessed the effect on AmiE expression by SDS-PAGE analysis of cell-free extracts (Figure 2). As has been previously shown [1–3], wild-type extracts showed clear induction of the 47 kDa acetamidase protein in the presence of acetamide. Mad1 showed a constitutively high level expression of AmiE. The identity of this protein was confirmed as the acetamidase by Western blotting (results not shown). Complementation with a functional copy of *amiA* (Mad1:pAGAN303) restored the ability of the cells to repress the acetamidase in the absence of an inducer. Since the acetamidase is not repressed in the absence of *amiA*, we conclude that it is a negative regulator of expression.

Multiple promoters are involved in acetamidase expression

Previous work has demonstrated that an inducible promoter was located in the 1.4 kb region approximately 1.5 kb upstream of amiE[3], although the possibility of more than one promoter was not excluded. In order to determine whether multiple promoters were involved in AmiE expression, we cloned the regions immediately upstream of each gene into a promoter probe vector carrying a β-galactosidase reporter gene (Table 1 and Figure 3). These constructs were first assayed for promoter activity in the wild-type strain. Four inserts with promoter activity were identified (Table 2). P_c drives the expression of amiC and is active at a low, but constitutive level. P_2 upstream of amiD is a stronger promoter which shows a two-fold induction in the presence of acetamide ($p < 0.012$ using the students t-test). This is consistent with the previous results which showed that a two-fold inducible promoter was located in the 1.4 kb upstream region [3] (although the exact location of the promoter was not defined within this region). P_1, upstream of amiA, had the highest promoter activity, but was constitutive. P_3 is a very weak promoter found immediately upstream of amiE itself, which could allow the transcription of a monocistronic message encoding the acetamidase only, whereas P_1 and P_2 are more likely to be responsible for polycistronic messages. Northern blotting has previously shown that a 1.2 kb acetamidase transcript is indeed present (and induced) in the cells [3]. This could either arise from P_3 or from processing of mRNA transcribed from P_1 or P_2.

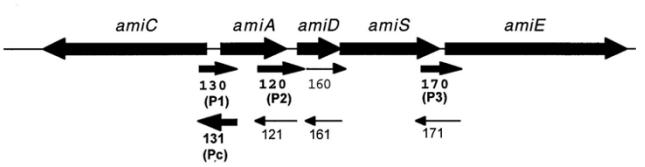

Figure 3
Promoter activity assays. The fragments assayed for promoter activity are shown, with arrows indicating the direction of the fragment realtive to the β-galactosidase gene. The pAGAN construct series numbers are given below each arrow. The four identified promoter fragments (P_c, P_1, P_2 and P_3) are labelled and indicated by thicker arrows.

Since all the fragments were small, they may not have contained all the regulatory sites normally present for each promoter. This may explain why we did not isolate a highly inducible promoter (only two-fold induction for P_2) and the fact that the P_3 promoter seemed to be very weak. Although we expected to find a highly inducible promoter, it is possible that one does not exist and that the induction of the acetamidase enzyme seen in the cell-free extracts may be a result of other mechanisms, such as an increase in mRNA stability.

There is a predicted consensus promoter sequence in the P_2 region which has both the -10 and -35 boxes of classical promoters [2]. None of the other promoter fragments show any similarity to previously identified promoter consensus sequences. Mycobacterial promoters often have a different structure from the typical *Escherichia coli* promoter [4,5], so this is not surprising. In addition, regulated promoters often do not have strong consensus sequences and rely on the presence of other regulatory or accessory proteins to recruit RNA polymerase and initiate transcription.

Promoter activity in Mad1

Three of the promoters were assayed for activity in the *amiA* deletion strain (Figure 3 and Table 2). P_1 promoter activity was unaffected (Table 2). P_2 activity was unchanged under induced conditions, but was significantly higher in the uninduced conditions ($p < 0.02$). The activity of P_c was also affected and was higher under both conditions ($p < 0.03$). Thus AmiA appears to repress both P_c and P_2 promoters.

The role of AmiA

Taken together the data suggest that AmiA acts to repress the P_2 promoter and thus prevent expression of the acetamidase. Since AmiA shows homology to the MarA DNA-binding group of regulatory proteins, it seems likely that it would exert this effect by binding to the P_2 promoter region and preventing transcription.

Conclusions

We have demonstrated that acetamidase expression is derepressed in an *amiA* deletion mutant and that of four promoters identified, two show increased activity in this strain. In light of this evidence we propose the following model. In the absence of acetamide, AmiA binds to the P_2 promoter region and prevents transcription of the three genes downstream. The small basal level of acetamidase expression arises from P_2 leakage, or from the P_3 promoter. AmiA may also bind to the Pc promoter and reduce expression of AmiC. In the presence of acetamide, AmiA no longer binds to the P_2 promoter region and transcription can then proceed at a higher level. In the latter case, it is probable that AmiC, which has a probable acetamide-binding domain [6], binds to both acetamide and AmiA thereby preventing its interaction with the promoters.

Table 1: Promoter assay constructs.

pAGAN number	pJEM number	Primer name	Primer sequence	PCR product position
120/121	12	PACT4	GGATCCATCTACCGGACTGCC	1677–1986
		PACT5	GGATCCGAAGGGACCGCAGTG	
130/131	14/13	PACT6	GGATCCACCAGCCCGACG	1291–1536
		PACT7	GGATCCTGAGCGACGAGT	
160/161	13	PACT10	GGATCCCCGAGCGCGATG	2000–2254
		PACT13	GGATCCACGTAGAAGAGC	
170/171	12	PACT9	GGATCCCTCCTTGCACTC	2758–3026
		PACT12	GGATCCCGCCGTGATCGG	

The regions shown in figure 3 were PCR-amplified from genomic DNA and cloned into pGEM-T (Promega). Primers were designed to contain *Bam*HI sites at each end. The fragments were then excised as *Bam*HI fragments and cloned into one of the pJEM vectors to give in-frame fusions to the promoterless β-galactosidase gene. The position of the PCR product with respect to the complete acetamidase region is given (assembled from Accession numbers U63095 and X57175).

Table 2: Promoter activity assays.

Construct	Promoter	Wild-type MM-S	Wild-type MM-AS	Mad1 MM-S	Mad1 MM-AS
pAGAN120	P_2	110 ± 34	169 ± 40	332 ± 110	301 ± 256
pAGAN130	P_1	705 ± 151	671 ± 109	970 ± 278	1079 ± 444
pAGAN131	P_c	20 ± 2.4	22 ± 6.8	41 ± 17	86 ± 46
pAGAN170	P_3	2.4 ± 0.5	4.1 ± 1.2	nd	nd
pJEM control	none	0.8 ± 0.4	1.3 ± 0.1	1.3 ± 0.2	2.2 ± 1.5

The constructs from figure 3 were assayed for β-galactosidase activity in both the wild-type and Mad1 strains. Values represent the mean ± standard error of six values from three independent transformants and are expressed as nmol O-nitro phenyl galactoside produced per min per mg total protein. Constructs pAGAN121, 160, 161 and 171 were all negative. nd – not determined.

Although the inducible acetamidase system has been used for genetic manipulation of mycobacteria [7–9], including over-expression of heterologous proteins, the system is far from ideal. Thus the characterisation of the role of *amiA* is an important step towards our understanding of the system and will allow the construction of more sophisticated systems in the future.

Material and Methods

Media

M. smegmatis was grown in Lemco medium (5 g/L Lemco powder, 5 g/L NaCl, 10 g/L Bacto peptone) with 0.05 % Tween 80 (liquid) or 15 g/L Bacto agar (solid). Kanamycin was added to 20 μg/ml, hygromycin to 100 μg/ml, gentamicin to 10 μg/ml and sucrose to 5% where appropriate. Minimal media [2] contained 0.05% Tween 80 and carbon sources (acetamide or succinate) at 0.02%.

Construction of deletion mutant strain Mad1

A suicide (non-replicating) delivery vector (pURR541) was constructed using a rapid cloning system [10]. First, the 1.4 kb *Bam*HI-*Sal*I and 0.5 kb *Sal*I-*Pst*I fragments indicated in Figure 1 were cloned into the manipulation vector p2NIL. The *sacB* and *hyg* marker genes were then cloned in as a *Pac*I fragment from pGOAL13 [10]; these confer sucrose sensitivity and hygromycin resistance respectively. The final delivery vector also had a kanamycin resistance gene. *M. smegmatis* Mad1 was constructed using a two-step strategy. The delivery vector pURR541 was electroporated [11] into *M. smegmatis* mc^2155 and single crossovers selected on hygromycin, kanamycin plates. One such transformant was streaked out to allow the second recombination event to occur. Double crossovers were identified by selecting for sucrose resistance and screening for kanamycin and hygromycin sensitivity. Southern blotting was used to determine which of the double crossovers had the wild-type genotype restored and which had the *amiA* deletion in the chromosome.

Complementation of Mad1

The complementing vector pAGAN303 was constructed by PCR-amplifying the *amiA* gene (Figure 1) using primers which were designed to contain *Eco*RI restriction sites. The PCR fragment was cloned as an *Eco*RI fragment into integrating vector pINT3 which carries the mycobacteriophage L5 integrase and attachment sites and a gentamicin resistance gene. Mad1 was electroporated with this plasmid and transformants selected on gentamicin.

Preparation of cell-free extracts

Strains were grown overnight in 5 ml Lemco broth and used to inoculate 100 ml of minimal media plus either acetamide and succinate (MM-AS) or succinate alone (MM-S) and incubated for 24 hours. Bacteria were harvested, washed and resuspended in 1 ml of 10 mM Tris-HCl pH 8. An equal volume of 0.1 mm glass beads were added and the suspensions subjected to 2 × 1 min pulses in the MiniBead Beater (Biospec Products). Cell debris was removed by spinning at 13000 g for 5 min.

Promoter activity assays

Promoter constructs (Table 1 and Figure 3) were electroporated into *M. smegmatis* and transformants selected with kanamycin. For each construct three transformants were grown up in 5 ml Lemco and used to inoculate 5 ml of MM-AS or MM-S. Cultures were incubated for 24, harvested, washed once and resuspended in 1 ml of 10 mM Tris-HCl pH8. Cell lysates were prepared by sonication with a 3 mm microprobe at 20% amplitude for 30 seconds using an Ultrasonic Processor (Sonics and Material). β-galactosidase activity was measured as previously described [12].

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