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Opposing effects of histidine phosphorylation regulate the AtxA virulence transcription factor in *Bacillus anthracis*

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Abstract

Expression of genes for *Bacillus anthracis* toxin and capsule virulence factors are dependent upon the AtxA transcription factor. An *atxA*-null mutant is avirulent in mice and is severely affected in toxin and capsule synthesis. The mechanism by which AtxA regulates the transcription of its target genes is unknown. In this report we show that AtxA is regulated by phosphorylation/dephosphorylation of conserved histidine residues within PTS (phosphoenolpyruvate:sugar phosphotransferase system) regulation domains (PRD). By means of amino acid substitutions that mimic the phosphorylated (H to D) or the unphosphorylated (H to A) state of the protein, we showed that phosphorylation of H199 of PRD1 is necessary for AtxA activation while phosphorylation of H379 in PRD2 is inhibitory to toxin gene transcription. Experiments of *in vivo* labeling with radioactive phosphate allowed us to conclude that H199 and H379 are the only AtxA residues subject to regulated phosphorylation. Our results link virulence factor production in *B. anthracis* to carbohydrate metabolism and, for the first time, provide a mechanistic explanation for AtxA transcriptional activity.

Introduction

The established virulence factors of the Gram-positive human and animal pathogen *Bacillus anthracis* are the toxin and the capsule. The anthrax toxin is a three-part toxin secreted by the bacterium consisting of protective antigen (PA), lethal factor (LF) and edema factor (EF). The genes encoding PA, LF and EF (*pagA*, *lef* and *cya*, respectively) are carried by the *B. anthracis* pXO1 virulence plasmid (Okinaka *et al.*, 1999). The capsule is poly-γ-D-glutamic acid and its synthetic operon (*capBCAD*) is localized on the pXO2 virulence plasmid (Makino *et al.*, 1988; Uchida *et al.*, 1993b). Toxin and capsule synthesis are both required for full virulence of *B. anthracis* (for a review see Mock and Fouet, 2001).

Virulence-factor gene expression in *B. anthracis* is triggered by specific cultural conditions in the laboratory. Capsule and toxin protein synthesis is induced when cells are grown in defined media, at 37ºC under high CO_2 tension and in the presence of bicarbonate (Bartkus and Leppla, 1989; Dai and Koehler, 1997; Koehler *et al.*, 1994; Leppla, 1988; Makino *et al.*, 1988; Sirard *et al.*, 1994). Essential for the transcription of both, the pXO1 borne toxin-encoding genes and the capsule biosynthetic operon on pXO2, is the product of the *atxA* gene also located on the pXO1 plasmid (Dai *et al.*, 1995; Drysdale *et al.*, 2004; Uchida *et al.*, 1993a; Uchida *et al.*, 1997). AtxA is a 475 amino acid protein (molecular weight 55.6 KDa), highly basic transcription factor. Its synthesis is affected by

growth temperature as six fold more protein was produced at 37ºC than at 28ºC; however, its synthesis was not affected by the presence of CO_2/bicarbonate (Dai and Koehler, 1997). An *atxA*-null mutant does not produce detectable levels of toxin and animals infected with this strain show a significantly reduced mortality and antibody response to the toxin proteins (Dai *et al.*, 1995). Similarly, capsule production was severely affected by an *atxA* deletion (Bourgogne *et al.*, 2003; Uchida *et al.*, 1997); however this effect is most likely mediated through the AtxA transcriptional regulation of two, pXO2-encoded, proteins, AcpA and AcpB (Drysdale *et al.*, 2004; Drysdale *et al.*, 2005; Uchida *et al.*, 1997; Vietri *et al.*, 1995). AcpA and AcpB share strong similarity with AtxA (26% and 25% identical residues, respectively) but they are not known to affect toxin synthesis (Bourgogne *et al.*, 2003).

In addition to being required for the expression of virulence factors, AtxA has been shown to be a global regulator of gene expression in *B. anthracis*. Transcriptional profiling has determined that AtxA regulates, directly or indirectly, the expression of numerous other genes on both plasmids and on the chromosome (Bourgogne *et al.*, 2003).

Despite the key role played by AtxA in the virulence of *B. anthracis* and the numerous phenotypic effects ascribed to this protein, nothing is known about the mechanisms involved in its transcriptional activity. Here we report that AtxA

activity is post-translationally regulated by phosphorylation/dephosphorylation of conserved histidine residues within PTS-regulated domains (PRDs).

PRDs are found in transcriptional antiterminators and activators, and are regulatory targets of the PTS systems (phosphenolpyruvate (PEP): sugar phosphotransferase systems) subject to phosphorylation or dephosphorylation in response to the availability of carbon sources (for reviews see: Barabote and Saier, Jr., 2005; Reizer and Saier, Jr., 1997; Stülke *et al.*, 1998; van Tilbeurgh and Declerck, 2001).

Results

AtxA contains PRD domains

The AtxA virulence factor is necessary for the transcription of the genes encoding the *B. anthracis* toxins (PA, LF and EF). However, the mechanism of AtxA activation of gene transcription is unknown. In an attempt to obtain insightful information, we analyzed the amino acid sequence of AtxA by means of BLAST and Pfam homology searches (www.sanger.ac.uk/software/Pfam). The results revealed that AtxA contains two PRD domains in addition to a previously identified DNA-binding domain and an unknown C-terminal domain (Fig. 1A). AtxA shared approximately 20% of identical residues with the Mga virulence gene regulator of group A streptococcus, also known to contain PRD domains (Kevin McIver, Functional Genomics of Gram-positive Microorganisms, San Diego, CA, 2005), but no significant homology scores were obtained with other PRD-containing proteins known to be regulated by histidine phosphorylation. Thus we carried out a Clustal alignment of the putative PRD domains in the AtxA protein with selected PRD domains (LicT, LevR, MtlR and Mga) and the results showed that, despite the lack of amino acid sequence conservation, a pattern of critical histidine residue positions was clearly noticeable (Fig. 1B) (Henstra *et al.*, 1999; Lindner *et al.*, 1999; Martin-Verstraete *et al.*, 1998; McIver and Myles, 2002). Conservation of histidine residues among characterized PRD-containing proteins allowed us to identify His199 of AtxA PRD1 and His379 of AtxA PDR2 as putative sites possibly subject to regulation by phosphorylation. An additional

histidine residue, His253 in PRD1, was also considered a possible target, despite its distance from the pattern of conserved histidine distribution, because of the corresponding misalignment of His199.

Phosphorylation of His379 inhibits AtxA activity

The histidine residue at position 379 in PRD2 of AtxA seemed to be in a highly conserved position among PRD domains of proteins known to be regulated by histidine phosphorylation. For this reason we used site-directed mutagenesis to mutate His379 to an alanine residue (thereby mimicking an unphosphorylated state) or to an aspartate residue (mimicking a phosphorylated state). The mutated *atxA* genes, as well as the wild type, were cloned in the multicopy vector pHT315 and transformed into a *B. subtilis* strain carrying the *pagA-lacZ* reporter in the *amyE* locus. The effect of the histidine substitution on AtxA protein expression and/or stability was assessed by means of a Western blot using a polyclonal antibody raised against purified AtxA. The results shown in Figure 2A indicated that the H379A and H379D substitutions did not affect the level of AtxA protein in the cells. The effect of these substitutions on *pagA* transcription in *B. subtilis* was assessed by means of β-galactosidase assays using the *pagA-lacZ* reporter. The results shown in Figure 3A indicated that the substitution of histidine with aspartate, H379D, completely blocked AtxA-dependent activation of *pagA* transcription while the H379A substitution did not affect the overall final level of transcription although it consistently caused a slightly reduced initial rate. In order to confirm that the histidine substitutions affected AtxA activity in *B.*

anthracis, the pHT315 derivative strains expressing the wild type and mutated proteins were transformed in the 34F2ΔatxA strain and the level of PA expressed in the supernatant was tested by Western blot analysis. The results shown in Figure 4A supported the conclusion that the H379D mutant protein no longer activated toxin production while the H379A was still active, although at a slightly lower level than the wild type protein.

These results indicated that phosphorylation of AtxA at histidine 379 was inhibitory to its activity as a *pagA* transcription factor.

Phosphorylation of His199 is required for AtxA activity

Since PRD-containing proteins are often regulated by phosphorylation events at two distinct sites, we examined the possibility that the PRD1 domain of AtxA could also contain histidine residues critical for activity. The H199 and H253 residues were each mutated to alanine or aspartic acid and the level of expression of the resulting AtxA mutant proteins was determined in the *B. subtilis* strain carrying the *pagA-lacZ* reporter fusion. As shown in Figure 2B, the level of AtxA protein expression obtained from the pHT315-derived constructs was essentially identical for the four mutants analyzed to the level observed with the wild type protein. Therefore we quantitated the level of *pagA* expression induced by each construct in *B. subtilis* by means of β-galactosidase assays. The results shown in Figure 3A indicated that the substitution of histidine 199 with alanine severely affected AtxA activity (6 fold) while the replacement with aspartate

resulted in a protein as active as the wild type or even more active. On the contrary, the H253A and H253D substitution did not affect *pagA* transcription (data not shown).

The pHT315 derivative plasmids carrying the H199A and H199D substitution were also transformed in the *B. anthracis* 34F2ΔatxA strain and Western blot analysis was carried out on culture supernatant using a polyclonal antibody raised against PA. The blot confirmed that, in *B. anthracis* as well as in *B. subtilis*, the amount of PA produced by the strain expressing AtxA H199D equaled the one produced in the control strain expressing the wild type AtxA protein; on the contrary, the strain expressing the AtxA H199A protein produced at least 50% less protein than the strain expressing wild type AtxA but still 3 times more than the control strain that did not express any AtxA (Fig. 4B).

Since the substitution of histidine with aspartate is believed to mimic a phosphorylation state, these results indicated that activity of AtxA is dependent upon phosphorylation on the histidine at position 199.

Inactivating substitutions have a dominant phenotype

In order to determine whether the phosphorylation state of one histidine residue had any effect on the activity of the other, we generated *atxA* mutant genes containing the four combinations of alanine and aspartate substitutions at the His199 and His379 residues. Four pHT315 derivate plasmids were obtained

carrying AtxA H199A-H379A, AtxA H199D-H379A, AtxA H199A-H379D, and AtxA H199D-H379D. The plasmids were transformed in the *B. subtilis* strain carrying the *pagA-lacZ* reporter fusion and in the *B. anthracis* 34F2ΔatxA strain. Western blot analysis on cell extracts of *B. subtilis* strains using the anti-AtxA antibody revealed that all double mutants were essentially expressed at the same level of the wild type protein (Fig. 2C). β-galactosidase activity of the *pagA-lacZ* reporter showed that the only double mutant protein maintaining some activity was the H199D-H379A combination. However, this strain expressed 3-5 fold less *pagA* than the strain carrying the wild type AtxA protein. The H199A-H379A expressing strain was able to transcribe *pagA* at a level slightly higher than the negative control strain not expressing AtxA; however, overall, 30 fold less β-galactosidase activity was detected compared to the positive control strain expressing the wild type AtxA protein.

These results indicated that AtxA histidine substitutions that inactivated the protein were dominant over the substitutions that maintain protein activity. This suggests that the two PRD regions may be interdependent in their effect on the overall activity of AtxA.

In vivo phosphorylation of AtxA

The mutagenesis studies suggested that phosphorylation of histidine residues may control the transcriptional activity of the AtxA virulence factor. In order to determine whether AtxA was indeed subject to phosphorylation, we carried out

an *in vivo* labeling experiment with radioactive phosphate followed by immunoprecipitation of AtxA with a specific antibody. The *B. anthracis* 34F2ΔatxA strains carrying the pHT315 plasmids expressing wild type or mutant AtxA proteins were grown in the presence of ^{32}P-H_3PO_4 as described in Experimental Procedures. Labeled cells were collected, lysed and subjected to immunoprecipitation of the AtxA protein using an anti-AtxA antibody. The immunoprecipitation samples were analyzed by SDS-PAGE and the results are shown in Figure 5. As expected the immunoprecipitated wild type AtxA protein was radioactively labeled confirming that phosphorylation is a post-translational event controlling its activity. The single alanine mutant substitutions of His199 and His379 also resulted in AtxA proteins that were still labeled while the double mutant protein, H199A-H379A was no longer labeled.

These results confirmed the genetic data indicating that double phosphorylation events occur at the H199 and H379 residues of AtxA and their effects are antagonistic on the activity of this transcription factor.

Experimental Procedures

Bacterial strains and growth conditions

E. coli K-12 TG1 and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. *E. coli* transformation was performed by electroporation using the Bio-Rad-Gene Pulser according to the supplier. Transformants were selected on LB broth supplemented with ampicillin (100μg/ml) or kanamycin (30μg/ml).

The *B. subtilis* strain used in this study was a derivative of JH642 (*trpC2 pheA1*), carrying a *pagA-lacZ* fusion. The *B. subtilis* strains were maintained on Schaeffer's sporulation medium supplemented with appropriate antibiotics at the following concentrations: kanamycin 2μg/ml; erythromycin and lincomycin, 5 and 25μg/ml, respectively.

B. anthracis 34F2 ($pXO1^+$ $pXO2^-$) and the derivatives of 34F2Δ*atxA* were grown in Schaeffer's sporulation medium supplemented with the appropriate antibiotics at the following concentrations: spectinomycin (100 μg/ml); erythromycin and lincomycin, 5 and 25μg/ml, respectively.

Plasmids and strains construction

The *pagA* transcriptional *lacZ* fusion was constructed in the promoterless vector pJM115 for ectopic integrations at the *amyE* locus of *B. subtilis* (Perego, 1993).

The *pagA* promoter region was amplified by PCR using the oligonucleotide primers Eco1 (5' AATTAGAATTCTTTAGCTTTCTGTA 3') and Bam0 (5' TAGATGGATCCCCTGTAGTAGAAG 3'), which introduced the EcoRI-BamHI restriction sites. Chromosomal DNA from *B. anthracis* strain 34F2 was used as template for the PCR reaction. The PCR product was digested with EcoRI and BamHI and cloned in pJM115. The construct was verified by DNA sequencing and was used to transform *B. subtilis* JH642 competent cells as described (Anagnostopoulos and Spizizen, 1961). Selection was performed on Schaeffer's sporulation medium with kanamycin. The correct integration of the pJM115 derivative at the *amyE* locus by a double-crossover event was confirmed by the *amyE*$^-$ phenotype on TBAB (Difco Triptose Blood Agar Base) plates containing 1% of starch.

The *atxA* gene was amplified by PCR using the following oligonucleotide primers: AtxA5'promEco (5' TATAAGAATTCTATGTTAATATGCT 3') and AtxA3'Bam (5' CAAATGGATCCAGGGCATTTATATTATC 3'). The PCR fragment was digested with EcoRI and BamHI and cloned in the multiple cloning site of the replicative vector pHT315 (Arantes and Lereclus, 1991). The *atxA* promoter region (210bp) was obtained by digestion of the above fragment with EcoRI and EcoRV followed by cloning in EcoRI-SmaI digested pHT315. The resulting constructs, pHT315-AtxA and pHT315-AtxPro, were used to transform the *B. subtilis* strains harboring the *pagA-lacZ* fusion or the *B. anthracis* 34F2ΔatxA strain. The transformants

were selected on Schaeffer's sporulation medium supplemented with erythromycin and lincomycin. Constructs were verified by sequence analysis.

Competent cells of *B. anthracis* were prepared following the method of Koehler *et al* (Koehler *et al.*, 1994).

Site-directed mutagenesis

For the mutagenesis on the H379 residue, the *atxA* coding sequence was PCR amplified using the primers: AtxANheI (5') and AtxA3'Bam (see above) and cloned in the NheI and BamHI sites of pET-28a (+). The resulting plasmid, pETAtxA, was used as a template for *in vitro* site-directed mutagenesis with the Muta-Gene kit (Bio-Rad). The mutagenic primers (in bold and underlined are the mutated nucleotides): AtxAH379A (5' GAGTTTCAAA**TGC**CATTGTTAAT 3') and AtxAH379D (5' GAG TTTCAAAAT**C**CATTGTTAAT 3') were used to introduce two point mutations that resulted in the replacement of the histidine residue at position 379 either by alanine or by aspartate yielding the constructs pETAtxAH379A and pETAtxAH379D respectively. These plasmids were digested with BclI and HindIII to release a 700bp fragment from the 3' end of the *atxA* gene carrying the corresponding point mutations. The fragments were transferred to pHT315-AtxA digested with BclI and HindIII thus resulting in the two plasmids pHT315-AtxAH379A and pHT315-AtxAH379D. For the mutagenesis of residues H199 and H253, site-directed mutagenesis of the *atxA* gene was carried out with the QuickChange® Site-Directed Mutagenesis Kit (Stratagene) according to the

manufacturer's protocol. The plasmid pHT315-AtxA was used as template. Four sets of oligonucleotides were used with the H199 codon (CAC) and H253 codon (CAT) changed to alanine (GCC) or aspartate (GAC) codons. The H199 oligonucleotides were (in bold and underlined is the triplet modified in the mutagenic oligonucleotides): (5'-GTACACCTATTCAAAA**CAC**AAATTGTGTGTTGTTCG-3' and 5'-CGAACAACACACACAATTT**GTG**TTTTGAATAGGTGTAC-3'). H253 oligonucleotides: (5'-CTTTCGGCGTTACATTA**CAT**GAAACTGAGATTTCATTTTTAG-3' and 5'-CTAAAAATGAAATCTCAGTTTC**ATG**TAATGTAACGCCGAAAG-3'). Double mutations of AtxA was also performed using pHT315-AtxA H379A and pHT315-AtxA H379D as templates and generated pHT315-AtxA (1) H379A+H199A, (2) H379A+H199D (3) H379D+H199A and (4) H379D+H199D. All constructs were sequenced to confirm the accuracy of the *atxA* gene and the expected mutations.

β-Galactosidase assays

B. subtilis strains harboring the *pagA-lacZ* fusion were grown at 37^{o}C in Schaeffer's sporulation medium supplemented with the appropriate antibiotics. β-galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (Ferrari *et al.*, 1986; Miller, 1972).

Preparation of samples for Western Blotting

B. anthracis 34F2Δ*atxA* cells carrying the pHT315-derived constructs were grown to OD_{600}=2.0 at 37°C in Schaeffer's sporulation medium supplemented with spectinomycin, erythromycin and lincomycin. Potato chymotrypsin protease inhibitor was added to 30 ml of culture media and the cultures were grown for 7 hr. Aliquots of 4 ml of the cell cultures were removed and centrifuged at 10,000 x g for 2 min at 4°C. The supernatants were filtered (Millipore filters, 0.2μm) and 1ml aliquots of the filtered supernatants were precipitated by adding 50μl of 5%TritonX-100 and 100μl of 100% trichloroacetic acid (TCA). The samples were mixed and incubated 10 minutes in ice. The precipitates were collected by centrifugation at 14,000 x g at 4°C for 5 min. The supernatants were removed and the pellets were washed 3 times by vortexing with 1ml cold acetone. The samples were centrifuged for 5 min at 16,000 x g after each washing. The pellets were air-dried, resuspended in 20μl of urea buffer (4M urea, 0.5% SDS, 100mM Tris-HCl pH 7.5) and stored at -20°C.

B. subtilis strains carrying pHT315-derived plasmids were also grown at 37°C in Schaeffer's sporulation medium supplemented with erythromycin and lincomycin. Aliquots of 3ml were removed and cells were harvested by centrifugation. Pellets were resuspended in a volume of lysis buffer (50mM Hepes pH 7.5, 50mM NaCl, 10mM $MgCl_2$, 10U DNase, 1mM PMSF, 1% Triton X-100) to obtain a final cell OD_{600}=1. Samples were sonicated 5 times for 10 seconds and 4xSDS loading buffer was added (8% SDS, 50% glycerol, 8% β-mercaptoethanol, 0.2M Tris-HCl pH 6.8) before electrophoresis or storage at -20ºC.

SDS-PAGE and Western analysis

5μl of 4xSDS loading buffer were added to the *B. anthracis* supernatant samples resuspended in urea buffer. The samples were then boiled for 3 min and 10μl of each sample were loaded on 10% SDS-PAGE gel. The *B. subtilis* lysate samples were loaded at 10μl per well on 10% SDS-PAGE gel. The gels were run at 30mA for 2 hr. The gels were stained with Coomassie to visualize the protein bands (not shown). For Western analysis 2μl of each *B. anthracis* supernatant sample were loaded on 10% SDS-PAGE gel and the gel was run as described above. The gel was transferred to a PVDF membrane (BioRad) in transfer buffer (Tris base 25mM, glycine 192mM, methanol 20%) at 20V overnight. The membrane was incubated 1 hour at room temperature in blocking buffer (5% dried milk in TBST (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 20)). The membrane was washed 5 times with TBST according to the protocol of the enhanced chemiluminescence light-based kit (ECL) from Amersham. The membrane was incubated for 1hr at RT with a polyclonal protective antigen antibody diluted 1:10,000 in blocking buffer. The blot was washed 5 times and was incubated for 1hr at RT with horseradish peroxidase goat anti-rabbit antibody (BioRad) diluted 1:10,000 in blocking buffer. Binding of the antibodies was probed with the ECL kit and the protein bands were visualized by PhosphorImager (Molecular Dynamics).

The Western analysis of the cell extracts from *B. subtilis* was performed similarly to that of the *B. anthracis* supernatant samples. The membrane was incubated for 1hr at RT with anti-AtxA rabbit antibody diluted 1:5,000 in blocking buffer. The following steps were carried out as described above.

In vivo phosphorylation and immunoprecipitation

B. anthracis 34F2Δ*atxA* strains containing pHT315-derived plasmids were grown at 37ºC in 10 ml cultures of LB broth containing erythromycin at 5 μg/ml and lincomycin at 25 μg/ml to an optical density at 600 nm of approximately 1.0. 1 mCi of ^{32}P-H_3PO_4 (MP Biomedicals, Solon, OH) was added to 4 ml of culture for each strain, and cultures were incubated for an additional 30 minutes at 37ºC. Labeled cells were collected by microcentrifugation, and pellets were resuspended in lysis buffer consisting of 300 μl of 10 mM Tris-HCl pH 8.0 containing 50 units of mutanolysin (Sigma-Aldrich, Saint Louis, MO). Following a one hour incubation in lysis buffer at 37ºC, approximately 50 mg of Glass Bubbles B38/4000 (3M, Saint Paul, MN) were added to each tube, and tubes were vortexed for 5 minutes at room temperature. Disrupted samples were microfuged for 10 minutes to remove cell debris and Glass Bubbles from supernatants. Two microcentrifugation steps were necessary to completely remove debris. Supernatants were pre-cleared by the addition of 50 μl of rProtein G Agarose slurry (Invitrogen, Carlsbad, CA) resuspended in 0.5% BSA/ddH_2O for 15 minutes followed by microcentrifugation. Supernatants were then split between labeled Eppendorf tubes and volume brought up to 500 μl total with

complete RIPA buffer (50 mM Tris-HCl [pH8.0], 150 mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% Sodium deoxycholate, 1x protease inhibitor cocktail). As indicated, 10 μl of rabbit polyclonal α-AtxA serum or 10 μl of rabbit pre-immune serum were added. Tubes were incubated overnight at 4ºC with constant rocking. The next morning, 100 μl of rProtein G Agarose slurry was added to each tube, and tubes were incubated for 5 hours at 4ºC with constant rocking. Samples were microfuged for one minute at 4ºC and supernatants removed. rProtein G Agarose pellets were washed a total of five times in 500 μl complete RIPA followed by microcentrifugation for 1 minute at 4ºC. The final washed pellets were resuspended in 100 μl of sample buffer (50 mM Tris-HCl [pH7.5], 10% glycerol, 2% SDS, 1% 2-mercaptoethanol, 0.1% Bromphenol blue) and heated to 65ºC for 5 minutes. Samples were then resolved by 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was dried and exposed to a PhosphorImager plate. Plates were scanned using a Molecular Dynamics Storm 840, and the data was analyzed using ImageQuant software.

AtxA purification and antibody production

Purification of AtxA from an overexpressing *E. coli* system will be described elsewhere (Tsvetanova *et al.*, in preparation). The protein was used to immunize a rabbit following standard protocols.

Discussion

We have demonstrated that the AtxA transcription factor, essential for the pathogenicity of the anthrax-causing agent *B. anthracis*, is post-translationally regulated by phosphorylation/dephosphorylation at two conserved histidine residues. The H199 and H379 of AtxA are located within PRD modules, PRD1 and PRD2 respectively, which structurally characterize the central domain of AtxA (Fig. 1). By means of amino acid substitutions that mimic phosphorylation (aspartate) or unphosphorylation (alanine) we have inferred that, while phosphorylation of H199 is stimulatory to AtxA activity, phosphorylation of H379 results in the absence of transcription from the AtxA-target promoter *pagA*.

Antagonistic effects of protein phosphorylation at distinct histidine residues of PRD domains is a common regulatory mechanism described for a variety of PRD-containing proteins regulated by PTS systems. PTS systems are responsible for the uptake and concomitant phosphorylation of a number of sugars in both $Gram^+$ and $Gram^-$ bacteria (Barabote and Saier, Jr., 2005). The phosphorylation cascade from PEP to sugar involves two energy-coupling proteins, Enzyme I (EI) and a histidine-containing phosphoprotein, Hpr. Several sugar-specific Enzyme IIs (EII) are required for the phosphorylation chain to proceed to the incoming sugar. EIIs are multidomain proteins organized in a single polypeptide chain or in individual polypeptides. EIIs are generally organized in three domains: EIIA and EIIB are involved in phosphoryl transfer,

while EIICs (and EIIDs where present) are membrane bound and catalyze sugar transport. In addition to its function in sugar transport, PTS is involved in a variety of regulatory mechanisms such as carbon catabolite repression, nitrogen metabolism, chemotaxis, competence, etc. (Barabote and Saier, Jr., 2005; Postma *et al.*, 1993; Reizer and Saier, Jr., 1997; Stulke and Hillen, 1998).

PTS regulation of carbohydrate metabolism occurs via phosphorylation or dephosphorylation of PRD-containing proteins. These proteins are either transcriptional antiterminators , thus binding RNA, or transcriptional activators binding DNA. PRD-containing proteins are found in most $Gram^+$ and some $Gram^-$ bacteria. Beside the BglG antiterminator of *E. coli* (Amster-Choder and Wright, 1990), the best characterized members of this family are from the genus *Bacillus*. For example the LicT, SacT and SacY antiterminators of *B. subtilis* or the LevR and MtlR activators of *B. subtilis* and *B. stearothermophilus*, respectively (Arnaud *et al.*, 1992; Henstra *et al.*, 1999; Lindner *et al.*, 1999; Martin-Verstraete *et al.*, 1998; Tortosa *et al.*, 1997). PRD-containing proteins carry two PRD domains, generally downstream of their RNA or DNA binding domain. Each PRD domain contains 2, more or less conserved, histidine residues. The first PRD domain (PRD1) is generally phosphorylated by a sugar-specific B domain of Enzyme II, while the PRD2 may be phosphorylated by Hpr. Opposing effects of EII-and Hpr-dependent phosphorylation have been observed in a variety of PRD-containing transcriptional regulators. Generally, phosphorylation of PRD1 by EII negatively affects the activity of the regulator

(Henstra *et al.*, 1999; Schmalisch *et al.*, 2003; Tortosa *et al.*, 2001). In contrast, the role of Hpr varies among different proteins: there is an absolute requirement for Hpr-dependent phosphorylation of PRD2 in certain regulators (LicT, SacT and, perhaps, BglG) while others are still active even in the absence of a functional Hpr (SacY and GlcT) (Amster-Choder, 2005; Arnaud *et al.*, 1992; Gorke and Rak, 1999; Lindner *et al.*, 1999; Lindner *et al.*, 2002; Schmalisch *et al.*, 2003; Tortosa *et al.*, 2001). Variations on these general concepts exists as no rule seems to apply for the physiological response associated with the phosphorylation in different PRD domains of any given PRD-containing regulator. Also variable is the number of histidine residues phosphorylated within each PRD domain (1 versus 2) and the significance of double phosphorylation when this occurs (Henstra *et al.*, 2000; Lindner *et al.*, 1999; Schmalisch *et al.*, 2003).

Our results indicate that AtxA is phosphorylated at two histidine residues, H199 in PRD1 and H379 in PRD2: a double mutant carrying alanine substitution at these positions was no longer labeled *in vivo* while each single alanine mutant still was (Fig. 5). This indicates that we have identified all phosphorylation sites in AtxA, at least for the assay conditions used in our study. The results also support our contention that the classical H✚A and H✚D mutations are mimicking phosphorylation/dephosphorylation.

The phenotypic effect of H199 and H379 substitution with the aspartate residue that mimics protein phosphorylation indicated that AtxA responds to

phosphorylation in an atypical manner. Phosphorylation of H199 in PRD1 was in fact required for transcriptional activation of the *pagA* target promoter while phosphorylation of H379 in PRD2 was inhibitory to this activity.

Additionally, each inactivating substitution (H199A and H379D) was negatively dominant over the activating substitution of the other histidine residue, suggesting that tight regulatory controls must exist in order for the bacterium to meet the conditions that allow AtxA to activate target gene transcription. Furthermore, a dual signaling pathway and interdependence between PRD1 and PRD2 must exist in order to explain the results obtained with the double histidine mutants. This regulatory control may be carried out by Hpr and any of the several Enzyme IIB subunits possibly encoded by the *B. anthracis* genome (Barabote and Saier, Jr., 2005), as expected if AtxA is indeed regulated by the PTS system like any PRD-containing protein so far characterized. The question then is whether AtxA activity is affected by the presence of carbohydrates in the growth medium. Preliminary data show indeed that in *B. anthracis*, the activity of AtxA is affected by carbohydrates (our unpublished data) and an investigation of the mechanisms and enzymes involved in this regulatory pathway is underway in our laboratory.

Phosphorylation of PRD domains has been shown to result in structural modifications that affect protein function. Genetic and structural analyses have shown that in LicT, phosphorylation of PRD2 provokes conformational changes

that stabilize the dimer form of the protein. Conversely, histidine substitutions that mimic phosphorylation in PRD1 leads to inactive and unstable proteins probably as a result of protein monomerization (Declerck *et al.*, 2001; van Tilbeurgh and Declerck, 2001). On the contrary, phosphorylation of PRD2 of BlgG by the EII enzyme BglF inhibited dimer formation and protein activity (Amster-Choder and Wright, 1992). An interdependence between the phosphorylation state of PRD1 and PRD2 has been proposed based on studies on SacY, LicT and MtlR (Henstra *et al.*, 2000; Tortosa *et al.*, 1997; van Tilbeurgh and Declerck, 2001). In fact, the structural characterization of LicT has shown that the phosphorylatable histidines of PRD1 and their counterparts in PRD2 are buried at the interior of the dimer interfaces making them inaccessible to phosphorylation or dephosphorylation by the regulatory enzymes. This results in an enhanced effect of the positive or negative role played by phosphorylation on one PRD module because it prevents the occurrence of the opposing effect on the other PRD module within a given regulator.

Interdependence between PRD1 and PRD2 may help explain the result we obtained with the AtxA double mutant H199D-H379A. Despite containing both substitutions that activate the transcription factor, this protein did not induce *pagA* transcription to the same level of the AtxA wild type protein (Fig. 3B). This could be due to an effect on protein conformation (monomer versus dimer) that would reduce the overall activity of the AtxA mutant within the cell.

The reported variability on structural conformation of PRD-containing proteins upon phosphorylation/dephosphorylation does not allow us to make predictions on the behavior of AtxA. The AtxA wild type protein has been isolated in the dimer form from an overexpressing *E. coli* strain and shown to bind to the promoter region of *pagA* (Tsvetanova *et al*., in preparation). No significant differences in structural conformation have been observed between the wild type and the H379A or the H379D mutant proteins. However, the latter mutant protein seems to be affected in binding the DNA target promoter, consistent with the lack of transcriptional activity shown in Figure 3 (our unpublished data). Further studies will be carried out on the H199 substituted proteins and the double mutants in order to define the role of phosphorylation/dephosphorylation on AtxA structural conformation and activity.

Our results may provide an explanation for the observation made by Dai and Koehler (Dai and Koehler, 1997) that overproduction of AtxA resulted in reduced expression of PA compared to the control strain. Independently of whether the active form of AtxA is dimer or monomer, overexpression of the protein from a relatively high copy number plasmid may result in titration of the activating signal and a reduced overall concentration of molecules that are capable of transcription activation.

The ability of AtxA to bind DNA is consistent with the presence of 2 helix-turn-helix (HTH) domains in its amino-terminal region (Fig. 1A). The presence of two

HTH domains is not uncommon in the DNA-binding motif of transcriptional activators in both prokaryotes and eukaryotes (Egan, 2002; Muller-Hill, 2006; Xu *et al.*, 1999), in contrast to the presence of only one RNA-binding domain, called CAT, in antiterminators (van Tilbeurgh *et al.*, 1997). The Pfam server places the two HTH domains of AtxA in the Mga family (McIver and Myles, 2002). Mga is also a transcription factor involved in virulence of *Streptococcus pneumoniae* that, similarly to AtxA, contains two PRD domains and may be subject to regulation by phosphorylation/dephosphorylation (Kevin McIver, Functional Genomics of Gram-positive Microorganisms, San Diego, CA, 2005). The mechanism of AtxA binding to DNA and activation of transcription is unknown. The promoter regions of genes known to be regulated by AtxA do not seem to share any sequence similarity suggesting that a mechanism independent of sequence recognition is in place. Consistent with this notion, we found that, in a DNase footprinting assay, AtxA protects an extended region of the *pagA* promoter, however protection was seen on DNA containing non AtxA-controlled genes as well, although the specificity for the latter was significantly lower (Tsvetanova *et al*., in preparation). Thus AtxA may require additional factor(s) to exert its function on specific promoters and/or the interaction with the transcription apparatus may be determinant for its activity.

The AtxA protein contains a C-terminal domain of approximately 90 residues that seems to be conserved only among AtxA-like proteins, according to the Pfam server. However, 25-40% of identical residues are observed with the linker

region connecting the PRD domain to an EIIA domain of putative transcriptional antiterminators of the BlgB-family (McClelland *et al.*, 2004) (GenBank accession number YP_153295). Whether this domain has any function in AtxA activity remains to be determined.

The AtxA-like proteins AcpA and AcpB (Drysdale *et al.*, 2004) may also contain PRD domains although they do not appear as clearly defined as in AtxA. A Pfam search identified only a PRD2 region in AcpA which contains an arginine residue at the position corresponding to H379 of AtxA. In AcpB, the Pfam search identified two PRD regions with a possible H196 in PRD1 corresponding to H199 of AtxA; in the PRD2 however, the H379 of AtxA is replaced by a tyrosine. PRD2 of AcpA and AcpB could have H326 and H327, respectively, possibly subject to regulated phosphorylation since they are in a relatively conserved position (Fig. 1C). Further studies will be required to determine whether these proteins are post-translationally regulated or they are simply under transcriptional control of AtxA.

The complexity in the regulation of expression of the genes for the anthrax virulence factors has been greatly increased by this revelation of the need for post-translational phosphorylation to control AtxA activity. These results provide the first mechanistic link between the environment and virulence gene expression.

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Figure Legends

Fig. 1: Structural organization and PRD domains in AtxA. **A:** Domain organization of AtxA according to the Pfam server. The HTH and Mga domains are helix-turn-helix DNA-binding regions, the latter with similarity to the Mga family of DNA-binding domain (McIver and Myles, 2002). The first and last residue of PRD1 and PRD2 are shown together with the amino acids surrounding the H199 and H379 residues. The carboxy-terminal domain is unique to AtxA-like proteins. **B:** Amino acid sequence alignment of PRD domains of representative proteins. Histidines known to be subject to phosphorylation are shown with the black background. In pair-wise alignments, the PRD1 and PRD2 of AtxA had the highest score with the MtlR PRD1 (22% and 13% identity, respectively). The alignment was carried out with the program Clustal W. No identical residue was found to be conserved among the sequences shown. Numbers refer to the position of the first and last residue shown, within each protein. MtlR is from *B. stearothermophilus*, LicT and LevR are from *B. subtilis*, and Mga is from *S. pneumoniae*. **C:** Amino acid sequence alignment of PRD1 and PRD2 of AtxA with putative PRD regions of the AtxA-like proteins AcpA and AcpB (Drysdale *et al.*, 2004). PRDs were identified by the Pfam server. The alignment was carried out with the Clustal W program; asterisk denotes identical residues. The highest scores in pair-wise alignments were between AcpAPRD2 and AcpBPRD2 (36% identity), AtxAPRD1 and AcpBPRD1 (20% identity), AtxAPDR2 and AcpAPRD2 (16% identity).

Fig. 2: Stability of AtxA mutant proteins. Western blot analyses carried out on *B. subtilis* cell lysates carrying plasmids pHT315 and its derivatives. **A:** Lane 1: Molecular weight markers in KDa (Magic Mark XP, Invitrogen); Lane 2: purified AtxA protein, 0.4µg; Lane 3: pHT315; Lane 4: pHT315-AtxA wild type; Lane 5: pHT315-AtxA H379A; Lane 6: pHT315-AtxA H379D; Lane 7: pHT315-AtxA promoter; Lane 8: purified AtxA, 0.4µg. **B:** Lane 1: molecular weight markers as in A, Lane 1; Lane 2: pHT315-AtxA H199A; Lane 3: pHT315-AtxA H199D; Lane 4: pHT315-AtxA H253A; Lane 5: pHT315-AtxA H253D; Lane 6: pHT315-AtxA wild type. **C:** Lane 1: pHT315-AtxA wild type; Lane 2: pHT315-AtxA H199A H379A; Lane 3: molecular weight markers as in A, Lane 1; Lane 4: pHT315-AtxA H199D H379A; Lane 5: pHT315-AtxA H199A H379D; Lane 6: pHT315-AtxA H199D H379D.

Fig. 3: Analysis of *pagA* transcription in *B. subtilis* strains expressing wild type or histidine mutants of AtxA. Cells carrying a *pagA-lacZ* fusion construct were grown in Schaeffer's sporulation medium supplemented with erythromycin and lincomycin. β-galactosidase assays were carried out on samples taken at hourly intervals before and after the transition (T0) between exponential growth and stationary phase. Symbols in **A** are: -✚-: pHT315 (buried under other symbols along the X axis); -▲-: pHT315-AtxA wild type; -■-: pHT315-AtxA H199A; -▼-: pHT315-AtxA H199D; -◆-: pHT315-AtxA H379A; -●-: pHT315-AtxA H379D. Symbols in **B** are: -✚-: pHT315 (buried under other symbols along the X axis); -

▲-: pHT315-AtxA wild type; -▽-: pHT315-AtxA H199A H379A; -○-: pHT315-AtxA H199D H379A; -◇-: pHT315-AtxA H199A, H379D; -□-: pHT315-AtxA H199D H379D.

Fig. 4: Production of PA in *B. anthracis* strains expressing wild type and mutant AtxA proteins. Supernatants from strain 34F2ΔatxA expressing the wild type or mutant AtxA proteins were assayed by Western blot using an anti-PA polyclonal antibody. **A:** Lane 1: molecular weight markers as in Figure 2A; Lane 2: pHT315-AtxA H379D; Lane 3: pHT315-AtxA H379A; Lane 4: pHT315-AtxAPro; Lane 5: pHT315-AtxA wild type; Lane 6 pHT315. **B:** Lane 1: pHT315; Lane 2: pHT315-AtxA wild type; Lane 3: pHT315-AtxA H199A; Lane 4: pHT315-AtxA H199D; Lane 5: molecular weight markers as in Figure 2; Lane 6: pHT315-AtxA H199A H379A; Lane 7: pHT315-AtxA H199D H379A; Lane 8: pHT315-AtxA H199A H379D; Lane 9: pHT315-AtxA H199D H379D.

Fig. 5: *In vivo* phosphorylation of *B. anthracis* AtxA wild type and histidine mutants. Strain derivatives of 34F2ΔatxA expressing the wild type or mutant AtxA proteins were labeled with ^{32}P-H_3PO_4 as described in Experimental Procedures. AtxA was immunoprecipitated using an anti-AtxA polyclonal antibody. Cell lysates of strains expressing AtxA H199A (Lanes 1 and 2), AtxA H379A (Lanes 3 and 4), AtxA H199A H379A (Lanes 5 and 6) or the AtxA wild type (Lane 7 and 8) were incubated with (**+**) or without (**-**) the anti-AtxA antibody

(α-AtxA). The wild type lysate was also incubated with the pre-immunized rabbit serum (Lane 9). Lane 10 contains a control buffer with AtxA antibody only.

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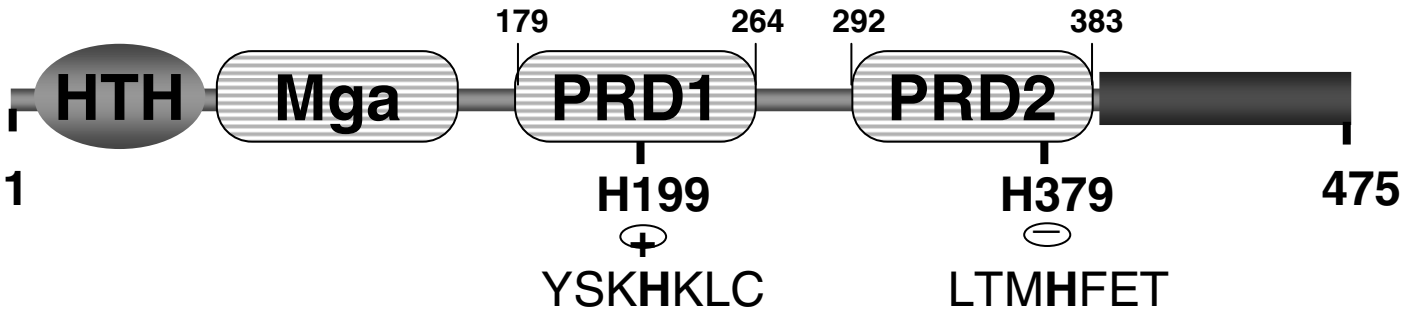
A



B

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LicTPRD1     56  -SEKFKTLLYDIPIECMEVSEEIIHYAKLQLGKKLN--DSIYVSLTDHINFAIQR-NQKGLDIKNA---LLWETKRLYKDEFAIGKEALVMVKNKTGVSLPEDEA-GFIAIHIVNAELNE------ 167
MtlRPRD1    193 LNTVTEKLLGLIDKKKLVTIEQQIERIKEELPFTIA--DSSYALVVHLALAIER-ISQGESINFD---QQYLETIQTTKEYETAEKIARSLEHAFRITIPKEEI-GYITMHLMGAKLRDRQGYML 309
AtXAPRD1    179 --------------------TDLINKMEKILNVQMY--TYSKHKLCVLFAITSR-LLSGNTIDNV---SGLILVNKNDDHYKTVASITSELQNSFGVTLHETEI-SFLALAL------------- 264
LicTPRD2    167 VNAELNEEMPNIIN-ITKVMQEILSIVKYHFKIEFNEESLHYYRFVTHLKFFAQR-LFNGTHMESQDDFLLDTVKEKYHRAYECTKKIQTYIERFYEHKLTSDEL-LYLTIHIERVVKQA------ 277
LevRPRD2    834 ----------LNPHHVIDMLLEWLQTVQDELGVIFN--NAVLIKVIMHTAFAFER-VIKQNPIAFLE---EEEINDQLKEMVYVTERTLAPYEEKLGLRISDDEK-LFIAAIFAEEVHGQLF---- 938
LevRPRD1    471 ----------FVEDDVIQMTKQLKEIAEHELDCTFD--RKFIYFLSMHIDAFLKR-GKQIDVLNTQ---ETDEIRDTHVKEYRVAMIFKDKIQEYFKVAIPEIEV-IYLTMLLHSIKSLKENKRVG 579
AtxAPRD2    292 --------------------KEITKGIEHKLQLGINYDESFLTYVVLIIKKALDKNFIQYYNYNIK---FIRHIKQRHPNTFNTIQECISNLNYTVYSHFDCYEI-SLLTMHFETQ---------- 383
MgaPRD1     198 ------------------------------HFT----LFQHLKILSGVNLIR-YYKGYSCSYNNK-KTSHRFSQLIQHSSEIQDLSRLFYLKFGLHLDEYTIAEMFSNHLNDKLEIG------ 278
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C

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AtxAPRD2    292 -----KEITKGIEHKLQLGINYDESFLTYVVLIIKKALDK-NFIQYYN-YNIKFIR--HIKQRHPNTFNTIQECISNLN--YTVYSHFDCYEISLLTMHFETQ 383
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AcpBPRD1    170 KKEVIFQYLGTLEESLGISL-----HVVSKRHLS--FFIAILLKRKQQGYKVQLNRKFLYFNTETPDYVKIGRIFEKLE--REFGVSLTVQDKILLTISI--- 260
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Fig.1

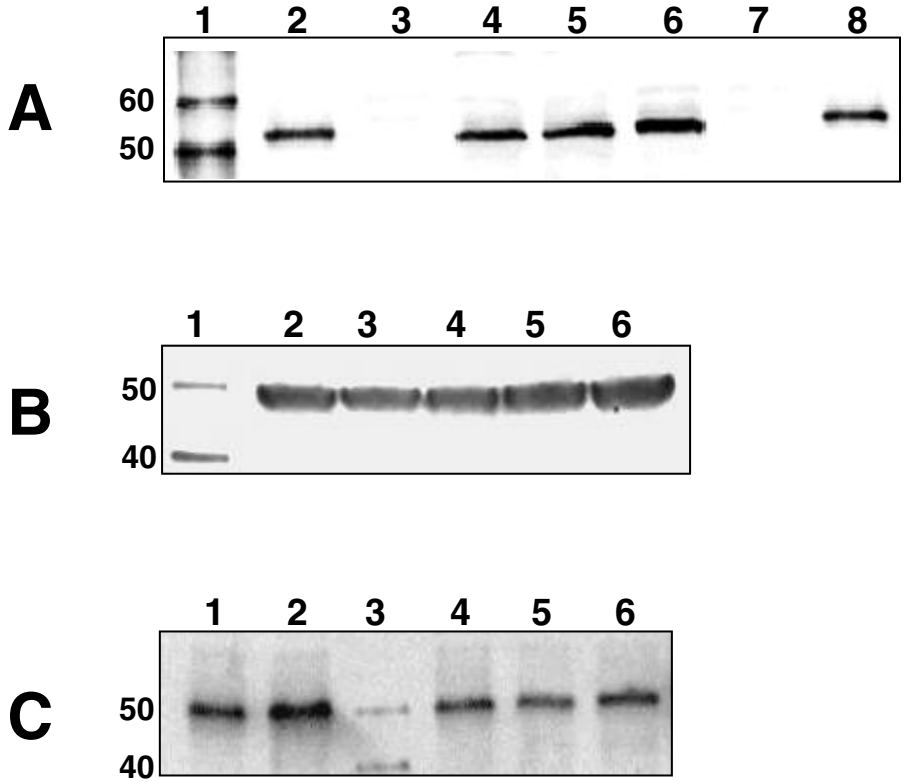

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Fig.2

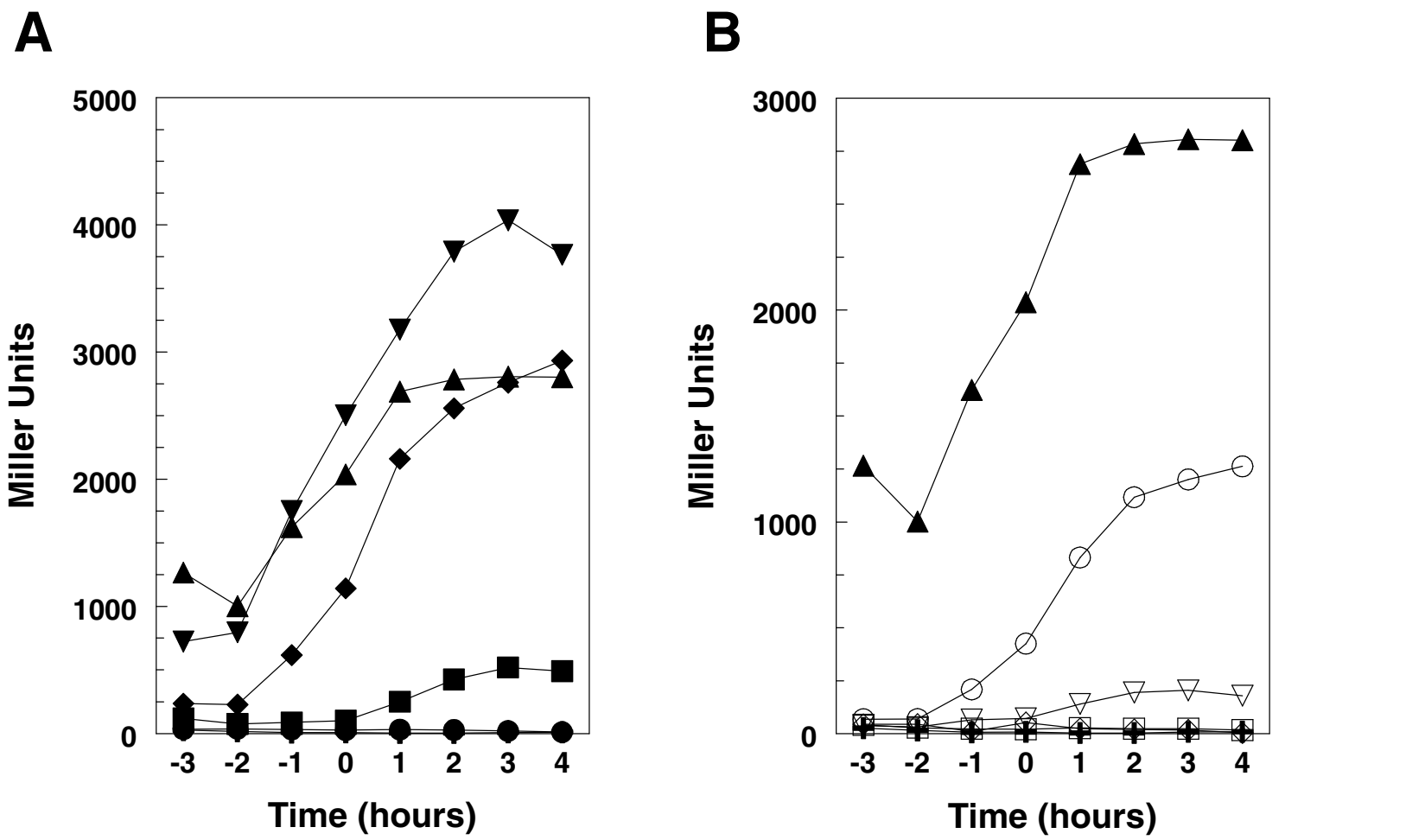

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Fig.3

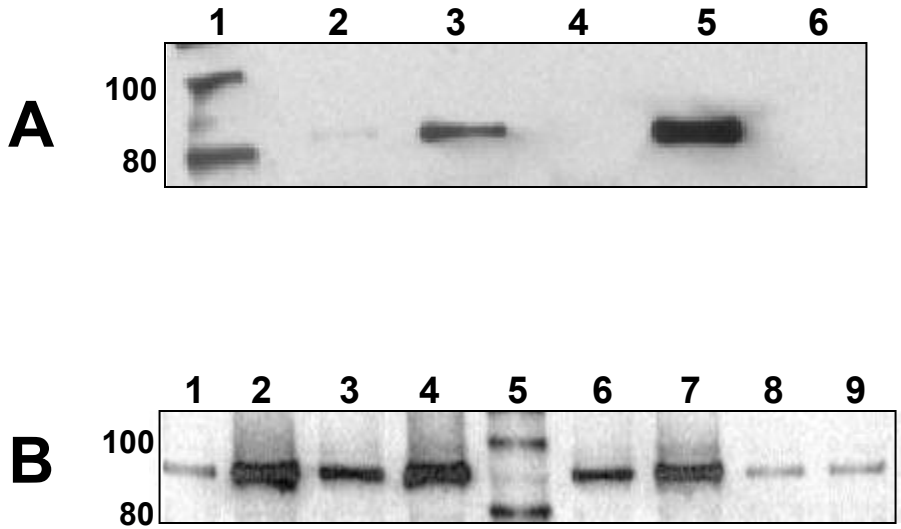

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Fig.4

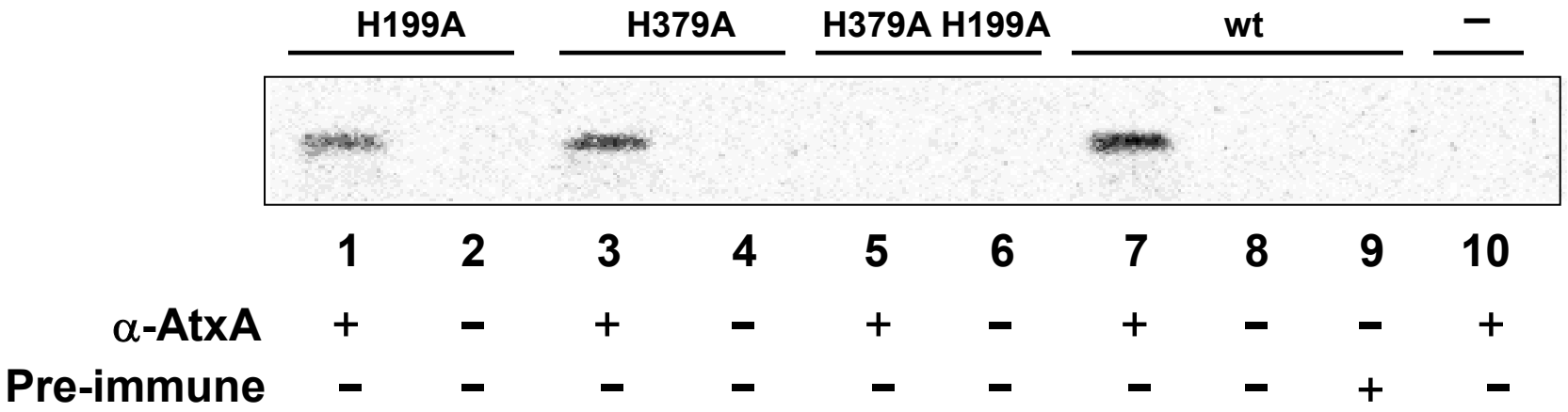

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Fig.5