Intrinsic curvature associated with the coordinately regulated anthrax toxin gene promoters

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The current model for virulence gene regulation in *Bacillus anthracis* involves several *trans*-acting factors, the most important of which appears to be the anthrax toxin activator encoded by the *atxA* gene. AtxA is a positive regulator of the toxin genes *pagA*, *cya* and *lef*, and of a number of other plasmid- and chromosome-encoded genes. The AtxA protein (56 kDa) possesses a predicted winged-helix DNA-binding domain and phosphotransferase system-regulated domains, but the mechanism for positive regulation of AtxA target genes is not known. Sequence similarities in the promoter regions of AtxA-regulated genes are not apparent, and recombinant AtxA binds DNA with a high affinity in a non-specific manner. We hypothesized that the toxin genes possess common structural features or *cis*-acting elements that are required for positive regulation. We employed deletion analyses to determine the minimal sequences required for *atxA*-mediated toxin gene expression. *In silico* modelling and *in vitro* experiments using double-stranded DNA corresponding to the toxin gene promoter regions indicated significant curvature associated with these regions. These findings suggest that the structural topology of the DNA plays an important role in the control of anthrax toxin gene expression.

INTRODUCTION

Coordinate control of virulence gene expression by many bacterial pathogens in response to host-derived signals is mediated by shared regulators. Common mechanisms of transcriptional control include activation or repression of transcription initiation by a protein that binds to promoter DNA sequences, and regulation of transcription termination by a protein or non-protein factor that controls formation of an RNA anti-terminator. In both mechanisms, binding of the regulator is attributed to recognition of specific nucleic acid sequences or structures associated with target genes or transcripts.

Anthrax toxin synthesis by *Bacillus anthracis*, the causative agent of anthrax, is dependent upon the presence of the regulator AtxA (Koehler *et al.*, 1994; Uchida *et al.*, 1993). Steady-state levels of mRNA transcripts associated with the three toxin genes *pagA*, *cya* and *lef*, are dramatically reduced in *atxA*-null mutants (Dai *et al.*, 1995). AtxA also affects transcription of the *B. anthracis* capsule biosynthetic operon *capBCADE*, in part via its positive control of the *cap* regulator *acpA* (Drysdale *et al.*, 2004). The anthrax toxin proteins and the *B. anthracis* capsule are considered

primary virulence factors of the bacterium. *B. anthracis* mutants devoid of the toxin and/or capsule genes are highly attenuated in some animal models. AtxA-mediated control of virulence during infection has also been established (Drysdale *et al.*, 2005). *atxA*-null mutants are highly attenuated and the antibody response to all three toxin proteins is decreased significantly in mice infected with an *atxA*-null mutant compared with those infected with the parent strain (Dai *et al.*, 1995).

Bioinformatic analysis of the predicted amino acid sequence of AtxA has revealed potential functional domains of the 56 kDa AtxA protein. AtxA is predicted to have an amino-terminal winged-helix DNA-binding domain. Nevertheless, specific nucleic acid-binding activity has not been reported. Recently, Tsvetanova *et al.* (2007) demonstrated that phosphorylation affects AtxA activity *in vivo*, suggesting that activation of AtxA-regulated genes is controlled by the phosphorylation state of AtxA. The predicted domains subject to phosphorylation bear the signature of phosphotransferase system-regulated domains (PRDs). Given that PRDs respond to carbohydrate availability (Stülke *et al.*, 1998; Stülke & Hillen, 2000), the presence of functional PRDs in AtxA indicates a mechanistic link between environmental signalling and virulence gene expression.

In addition to AtxA, other regulators have been identified that affect toxin gene transcription. These include the transition state regulator and DNA-binding protein AbrB

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A supplementary table listing the primers used in this study and a supplementary figure showing *in silico* models of other AtxA-regulated promoters are available with the online version of this paper.

(Saile & Koehler, 2002), and the alternative sigma factor for RNA polymerase, SigH (Hadjifrangiskou *et al.*, 2006). However, these regulators have been proposed to control expression of the *atxA* gene, rather than affect toxin gene expression directly. One regulatory protein, PagR, encoded by the second gene of the bicistronic *pagAR* operon, has been shown to bind to nucleic acid sequences in the promoter region of *pagAR* and function in weak autogenous control of the operon (Hoffmaster & Koehler, 1999; Mignot *et al.*, 2003). PagR also binds to sequences in the promoter regions of two other genes, *eag* and *sap*, which encode S-layer proteins that have not been associated with virulence. Although specific regions of each PagR-regulated promoter were protected in DNase I footprint analyses, common DNA sequences resembling an operator motif could not be identified (Mignot *et al.*, 2003).

Despite advances in knowledge of *trans*-acting regulators that affect transcription of *B. anthracis* virulence genes, a detailed analysis of the *cis*-acting features of AtxA-controlled genes is lacking. Microarray experiments employing parent and *atxA*-mutant strains have identified a number of *atxA*-regulated genes (Bourgogne *et al.*, 2003), expanding the role of AtxA to that of a global regulator. Nevertheless, no sequence similarities have been noted in the promoter regions of genes within the AtxA regulon. The 5' ends of mRNA transcripts associated with the most stringently controlled *atxA* targets, *pagAR*, *lef* and *cya*, have been mapped (Dai *et al.*, 1995; Koehler *et al.*, 1994). While the *lef* and *cya* genes each possess a single apparent transcription start site, *pagAR* transcription appears to start at two distinct sites. The major start site, P1, is tightly controlled by *atxA*, while the P2 site, located closer to the translational start codon for *pagA*, appears to be expressed constitutively at a relatively low level (Koehler *et al.*, 1994). AtxA-dependent transcription from the *pagAR* P1 site and the *lef* and *cya* start sites is induced during growth in elevated (5–20%) atmospheric CO₂ and/or in media containing dissolved bicarbonate (Bartkus & Leppla, 1989; Koehler *et al.*, 1994). The CO₂/bicarbonate signal may be relevant as a host signal during infection.

In addition to sequence-specific control sites recognized by *trans*-acting regulators, promoter activity can be affected by local DNA structure. Specific structural features such as dynamic 3D geometry, thermostability and responsiveness to superhelicity, play significant roles in the ability of a DNA sequence to respond to or allow protein binding. In prokaryotes, small nucleoid-associated proteins, such as H-NS, IHF and HU, associate with the chromosomal DNA, affecting DNA topology, and ultimately gene expression on a global level (Garcia *et al.*, 2007; Luijsterburg *et al.*, 2006). In addition, there are multiple examples of the influence of intrinsic curvature on gene expression wherein local topology affects binding to specific regulatory proteins and subsequent protein-induced bending (Owen-Hughes *et al.*, 1992; Rippe *et al.*, 1995; Yamada *et al.*, 1991; Zuber *et al.*, 1994). A well-studied example is the *Salmonella typhimurium* *put* operon for proline utilization

(Perez-Martin *et al.*, 1994a, b), wherein operator sites for binding of a regulatory protein are separated by a region of curved DNA, and removal of the curved DNA results in lower levels of repression (Ostrovsky de Spicer *et al.*, 1991). Also in *S. typhimurium*, osmoregulation of the *proU* promoter requires a curved sequence found 200 bp downstream of the transcription start site (Owen-Hughes *et al.*, 1992). The LrpC protein of *Bacillus subtilis* is a global regulator that recognizes specific DNA structure. LrpC complexes with curved sequences containing phase-A tracts to wrap DNA and form nucleosome-like structures (Beloin *et al.*, 2003).

To gain an understanding of the intrinsic properties of the anthrax toxin gene promoter DNA, we undertook a thorough characterization of the promoter regions. We hypothesized that the toxin gene promoters contain *cis*-acting regions with common sequence and/or structural features associated with *atxA*-dependent expression. In work reported here, we determined the minimal sequences of the toxin gene promoters that are required for *atxA*-dependent transcription, and employed *in silico* and *in vitro* analyses to demonstrate that the anthrax toxin promoters are characterized by intrinsic curvature.

METHODS

Strains and media. *B. anthracis* strain 7702 (Cataldi *et al.*, 1992) and an isogenic *atxA*-null mutant, UT60 (Koehler *et al.*, 1994), were used for this study. *Escherichia coli* strains TG1 (Zymoresearch) and GM2163 (New England Biolabs; NEB) were used as hosts for cloning. All strains were cultured in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989), unless indicated otherwise. *B. anthracis* cell extracts used for β -galactosidase assays were obtained from cells cultured with shaking at 37 °C in CaCO₃ medium [CA medium (Thorne & Belton, 1957) buffered with 100 mM HEPES (pH 8.0) and 0.8% (w/v) sodium bicarbonate], as described previously (Hadjifrangiskou *et al.*, 2006). Antibiotics were purchased from Sigma–Aldrich or Fisher Scientific, and were added to media at the following concentrations when appropriate: ampicillin, 100 μ g ml⁻¹; kanamycin, 100 μ g ml⁻¹; and erythromycin, 150 μ g ml⁻¹ for *E. coli* and 5 μ g ml⁻¹ for *B. anthracis*.

Extraction of chromosomal DNA from *B. anthracis* cultures was performed using the Mo Bio Laboratories genomic isolation kit. The Wizard SV Plus Mini Prep kit (Promega) was used for extraction of plasmid DNA from *E. coli*. *B. anthracis* was electroporated with unmethylated plasmid DNA from *E. coli* GM2163, as described previously (Koehler *et al.*, 1994). Recombinant DNA techniques were performed using standard procedures (Ausubel, 1993). Restriction enzymes and T4 DNA ligase were purchased from Promega and Fisher Scientific. *Taq* DNA polymerase was purchased from NEB and *Pfu* Turbo DNA polymerase from Stratagene. Plasmids created for deletion analyses of the *pagA*, *cya* and *lef* promoter regions are listed in Table 1. Supplementary Table S1 lists primers used in this study.

β -Galactosidase assays. Cells from 1 ml samples of cultures grown to late exponential phase were collected by centrifugation and soluble fractions were obtained as follows. Cell pellets were resuspended in PBS and mechanically sheared by using a Mini-BeadBeater-8 (MBB8) (Biospec Products) according to the manufacturer's protocol. Cell debris was removed by centrifugation at 16 000 g for 10 min and the soluble fraction was treated with 40 μ l 25 $\times$ Protease Inhibitor

Table 1. Plasmids constructed for promoter deletion analyses

Plasmid*	Promoter sequences present†	Primers‡
P_{pagAR}		
pUTE605	−77 to +88	MH59s/MH57as
pUTE606	−100 to +88	MH56s/MH57as
pUTE632	−90 to +88	MH58s/MH57as
pUTE638	−85 to +88	MH116s/MH57as
pUTE639	−80 to +88	MH117s/MH57as
pUTE686§	−90 to +78	MH58s/MH152as
P_{cya}		
pUTE624	−212 to +63	MH101s/MH102as
pUTE625	−152 to +63	MH103s/MH102as
pUTE626	−122 to +63	MH104s/MH102as
pUTE633	−101 to +63	MH111s/MH102as
pUTE637	−77 to +63	MH115s/MH102as
pUTE672	−122 to +15	MH104s/MH136as
P_{lef}		
pUTE621	−195 to +136	MH96s/MH99as
pUTE622	−121 to +136	MH98s/MH99as
pUTE623	−70 to +136	MH100s/MH99as
pUTE646	−87 to +136	MH119s/MH99as
pUTE648	−58 to +136	MH118s/MH99as
pUTE669	−70 to +81	MH100s/MH133as

*All PCR-generated fragments were cloned in the *Hind*III–*Bam*HI sites of the pHT304-18z reporter vector (Agaisse *et al.*, 1994).

†Number of base pairs upstream (−) and downstream (+) of the *atxA*-regulated transcription start.

‡Primer sequences are listed in Supplementary Table S1.

§Plasmids in bold type denote constructs containing minimal functional promoter regions.

Cocktail (Roche Applied Sciences). β -Galactosidase assays were performed according to Miller (1972). At least three independent cultures were evaluated for enzyme activity using duplicate samples from each culture. The figures show average promoter activity.

In silico promoter analysis. DNA sequences were submitted to the DNA tools servers ‘bend.it’ and ‘model.it’ (<http://hydra.icgeb.trieste.it/dna/>) (Munteanu *et al.*, 1998; Vlahovick *et al.*, 2003), and analysed for the presence of curvature using the Consensus (Trinucleotide) (Gabrielian & Pongor, 1996) and NMR (Dinucleotide) (Ulyanov & James, 1995) models. Figures show static 3D models generated using the model.it server based on the Consensus (Trinucleotide) model.

Circular permutation assays. Promoter regions of the toxin genes were amplified using 7702 DNA as template, *Pfu* Turbo DNA polymerase (Stratagene), and the primer pairs MH197s/MH198as (*P_{pagAR}*), MH201s/MH202as (*P_{cya}*) and MH199s/MH200as (*P_{lef}*) (see Supplementary Table S1). The *papR* (BA5594, AE016879:5080849–5080995) promoter region was amplified using MH205s/MH206as to generate the 169 bp region from −170 to −2 in relation to the predicted translational start site. The resulting amplicons were cloned into the *Xba*I–*Sal*I sites of pBend2 (Kim *et al.*, 1989) to generate plasmids pUTE750 (*P_{pagAR}*), pUTE755 (*P_{cya}*), pUTE749 (*P_{lef}*) and pUTE757 (*P_{papR}*). Plasmids were digested with the following enzymes individually: *Bgl*II, *Nhe*I, *Spe*I, *Xho*I, *Eco*RV, *Pvu*II and *Bam*HI. Electrophoresis of the digested fragments was

carried out in 5 and 8 % polyacrylamide gels, containing 10 % (v/v) glycerol in 1 × TBE as described elsewhere (Kim *et al.*, 1989; Sinden & Hagerman, 1984). DNA mobility was assessed at room temperature and at 4 °C. Experiments were performed four to six times for each promoter.

RESULTS

Deletion analysis of the toxin gene promoter regions

We performed deletion analysis to determine the minimal sequences of the toxin gene promoters that are required for AtxA-dependent transcription of the toxin genes. A series of fragments with successive 5′ truncations, as depicted in Fig. 1, was cloned upstream of a promoterless *lacZ* gene in pHT304-18z (Agaisse & Lereclus, 1994). Each construct was introduced into the parent strain 7702 (Cataldi *et al.*, 1992) and the isogenic *atxA*-null strain UT60 (Koehler *et al.*, 1994). β -Galactosidase activity was assessed following growth under conditions that favour toxin gene expression (CaCO₃ medium at 37 °C in an atmosphere containing 5 % CO₂) (Dai *et al.*, 1995). No significant promoter activity was detected in the UT60-derivatives, consistent with the requirement of *atxA* for toxin gene transcription.

The 7702-derived clones exhibited varying amounts of β -galactosidase activity. We determined that DNA sequences beyond 90 bp upstream of the major transcriptional start site P1 were not required for maximum *pagAR* promoter activity (Fig. 1a), corroborating and fine-tuning a previous report indicating that AtxA-dependent transcription of *pagAR* does not require DNA sequences more than 111 bp upstream of the P1 start site (Dai *et al.*, 1995). Promoter activity was abolished in a reporter construct harbouring only 77 bp of sequence 5′ of P1. Interestingly, the region implicated as being essential for *pagAR* transcription contains DNA sequences reported to be bound by the repressor PagR (Mignot *et al.*, 2003). In the case of the *cya* promoter, the minimal functional control region extended 122 bp upstream of the transcription start (Fig. 1b). Shortening of the 5′ end to 101 bp upstream of the start site reduced promoter activity approximately fivefold.

Analysis of the *lef* promoter region was less straightforward. AtxA-dependent expression of the *lef* promoter was evident in a construct harbouring only 70 bp upstream of the transcriptional start site for the gene (Fig. 1c). Yet constructs containing sequences from further upstream exhibited lower promoter activity. These results suggest that a repressor binding site may reside upstream of this region. Given that the transition state regulator AbrB has been shown to negatively regulate toxin gene expression (Saile & Koehler, 2002; Strauch *et al.*, 2005), we measured the β -galactosidase activity of parental and *abrB*-null strains carrying reporters containing 70 bp of DNA upstream of the transcriptional start site (the minimal functional sequence) and reporters containing 195 bp of DNA upstream of the transcriptional start site. Although

Fig. 1. Deletion analysis of the toxin gene promoters. Reporter vector constructs carrying successive 5' deletions of the (a) *pagAR*, (b) *cyA* and (c) *lef* promoter regions were introduced and tested in *B. anthracis* strain 7702 (Cataldi *et al.*, 1992). β -Galactosidase activity was measured for samples obtained during the late exponential phase of growth from cultures grown under toxin-inducing conditions. Grey bars represent promoter sequences tested. Arrows indicate the 5' ends (+1) of the AtxA-regulated transcripts. Numbers at the ends of the grey bars indicate the number of base pairs upstream (–) or downstream (+) of the AtxA-regulated transcriptional start in each construct. Translational start sites are indicated by vertical lines. Data shown represent the mean and SD of six independent experiments.

both constructs had higher overall activities in the *abrB*-null background, the fold-difference between them remained the same (data not shown), indicating that *AbrB* is not responsible for the low activity associated with the longer construct. To assess whether the shorter promoter fragment had lost its dependence on the presence of elevated CO_2 , we tested the β -galactosidase activity of parental strains carrying the two reporter constructs in the absence of elevated CO_2 . No significant activity was observed for either (data not shown).

The deletion analysis results indicated that the minimal functional promoter regions of the three toxin genes differ significantly in terms of overall sequence length and distance from the 5' ends of toxin gene mRNAs. To assess sequence similarities within the minimal functional promoter regions of the toxin genes, we generated alignments using CLUSTAL W (Chenna *et al.*, 2003; <http://www.ebi.ac.uk/clustalw/>). Each sequence aligned was terminated at the nucleotide corresponding to the transcriptional start site of each promoter. Our results did not reveal significant sequence similarities between the three sequences, as depicted in Fig. 2. Overall, the *pagAR*, *cyA* and *lef* promoter regions were highly A + T-rich (85, 79

and 80 % A + T, respectively) compared with the overall 64.6 % A + T content of the *B. anthracis* genome (Read *et al.*, 2003). The minimal functional promoter regions also had prominent stretches of A- and T-tracts; however, a thorough comparison of the frequency of A- and T- tracts throughout the *B. anthracis* genome was not performed. Given that A- and T-tracts are implicated in the formation of kinks and/or bends in DNA, we wanted to examine the degree of curvature in the identified minimal functional promoters.

In silico analysis of the toxin gene promoters

To examine the toxin gene promoter DNA for predicted intrinsic curvature and structural features that may be related to coordinate expression, we employed the results of a large-scale computational analysis performed on two sequenced strains of *B. anthracis* (<http://www.cbs.dtu.dk/index.shtml>) (Hallin & Ussery, 2004; Pedersen *et al.*, 2000) and methods available from the DNA tools web servers bend.it and model.it (<http://hydra.icgeb.trieste.it/dna/>) (Munteanu *et al.*, 1998; Vlahovicek *et al.*, 2003). Modelling of the promoter sequences was performed using

PpagA (-90to+1)	AATTCTTTTTTATGTTATATATTTATAAAAGTCTGTTTAAAAAGCCAAAAATAAATAAT	60
Plef (-70to+1)	-----AAGAAA	6
Pcya (-122to+1)	-----GATTATATTTGTAAATAAAATTGTAATTTAACATGTAGAATAAGAGA	48

PpagA (-90to+1)	TATCTCTTTTTATTTATA--TTATATTGAACTAATCTTTTT-TATGTTATATATTTA	116
Plef (-70to+1)	TATTGCCTTTAATTTATG--AGGAAATAAGTAAAATTTTCTACATACTTTATTTTATTG	63
Pcya (-122to+1)	TTTTTAGTTTATTAAACAGGATGAAAATCCATAAAACCGTAAATGTGATTTCTAAATTAG	108
	* * *** * * * * * * *	
PpagA (-90to+1)	TAAAAGTCTGTTTAAAAAGCCAAAAATAAATAATTATCTCTTTTATTTATATATTAT	176
Plef (-70to+1)	TTGAAATG-----	71
Pcya (-122to+1)	TTTAAATAAAAAC-----	123
	* **	
PpagA (-90to+1)	GAAACT 182	
Plef (-70to+1)	-----	
Pcya (-122to+1)	-----	

Fig. 2. Sequence alignments of the toxin gene promoters. DNA sequences corresponding to the minimal functional promoter regions for *pagAR*, *lef* and *cya* were compared using CLUSTAL W (Chenna *et al.*, 2003; <http://www.ebi.ac.uk/clustalw/>). Asterisks indicate identical nucleotides. Nucleotides corresponding to the 5' ends of *atxA*-regulated transcripts are underlined.

the Consensus (Trinucleotide) and NMR (Dinucleotide) scales, as these have been shown experimentally to be the most reliable for predicting DNA curvature (Gabrielian & Pongor, 1996). Similar results were obtained using either property scale. Only the consensus bendability data are presented here.

3D models of the toxin gene promoters indicate common structural characteristics. For each promoter, a region of high curvature precedes the *atxA*-dependent transcription start. In the models shown in Fig. 3(b), the transcription start site of each promoter is in the same plane as an apparent kink. In the cases of the *pagAR* and *lef* promoters, the kink is located immediately downstream of the transcriptional start, while in the *cya* promoter the kink is located upstream.

Models of the extended *pagAR* and *cya* promoter regions show an additional bend, which is not apparent in the corresponding *lef* promoter region. Our modelling analyses predicted differences in the conformation and positioning of the common structural features in the non-functional 5'-deletion fragments. The modelled non-functional *lef* and *cya* promoter sequences are devoid of the high curvature preceding the transcription start, while the corresponding *pagAR* promoter sequence is predicted to have altered positioning of this bend (Fig. 3a, 5'-deletion non-functional sequences).

In addition to the toxin genes, AtxA has been reported previously to influence expression of a number of other *B. anthracis* genes (Bourgogne *et al.*, 2003). We modelled the promoter regions of two additional genes, pXO1-90 and pXO1-91, which are positively regulated 61-fold and 25-fold, respectively, by AtxA (Bourgogne *et al.*, 2003). Models generated for these promoter regions shared the same characteristics as those identified for the toxin gene promoters (Supplementary Fig. S1), further supporting

the hypothesis that local DNA structure is associated with regulation by AtxA.

In order to verify that the predicted structural features are unique to promoters of genes highly regulated by AtxA, we modelled a series of other DNA sequences of similar A+T content. Such sequences included promoter regions of non-AtxA-regulated genes on the *B. anthracis* plasmids and chromosome, as well as random intergenic regions. Our results revealed that the majority of modelled sequences were predicted to contain areas of high curvature; however, the identified structural features did not resemble those predicted for the toxin gene promoters (Fig. 3b).

Gel mobility assays indicate curvature of toxin gene promoters

Comparison of the minimal functional promoter regions using *in silico* analysis revealed that each promoter is characterized by regions of high curvature. DNA mobility in polyacrylamide gels is affected by DNA structure, in particular DNA curvature (Chastain *et al.*, 1995; Hagerman, 1990). The rate of migration depends upon the angle and position of the DNA curves, as well as the physical properties of the gel (Chastain *et al.*, 1995; Ostrovsky de Spicer *et al.*, 1991; Ostrovsky de Spicer & Maloy, 1993). Curved DNA migrates aberrantly in non-denaturing polyacrylamide gels. Generally, bent DNA migrates more slowly with increasing concentration of polyacrylamide compared with non-curved sequences of the same length, but in some cases intrinsic DNA structure causes DNA to migrate faster in high-percentage polyacrylamide gels (Drak & Crothers, 1991; Matsugami *et al.*, 2006; Ohya *et al.*, 1998; Schroth *et al.*, 1992). To test for the presence of curvature, we examined the mobility of the minimal functional promoter regions in 5 and 8% polyacrylamide gels. Fig. 4 is a schematic representation

Fig. 3. Modelling of the toxin gene promoters. *In silico* analysis was performed using the consensus (Trinucleotide) property scale (Gabrielian & Pongor, 1996) of the bend.it DNA analysis server (DNA tools; <http://hydra.icgeb.trieste.it/dna/>) (Munteanu *et al.*, 1998; Vlahovicek *et al.*, 2003). Three different lengths for each promoter were selected based on deletion analysis results. (a) Modelling of the *pagAR*, *lef* and *cya* promoter regions from top to bottom. Top, full-length promoter sequences, corresponding to the longest fragments tested by using β -galactosidase reporter genes. Middle, 5'-deletion analysis non-functional promoter sequences. Bottom, minimal functional promoter sequences. In each panel, the purple arrows mark the nucleotides corresponding to the 5' ends of *atxA*-regulated transcripts. Black asterisks indicate the locations of kinks. Negative numbers indicate number of base pairs upstream of each *AtxA*-regulated transcription start; positive numbers indicate number of base pairs downstream of the transcription start. (b) Modelling of the *papR*, BA1625 and *bla1* promoter regions.

indicating the location and size of predicted bends (black bars) in each digestion fragment.

We cloned the minimal functional toxin gene promoter regions and the *papR* promoter region into the *XbaI*–*SalI* restriction sites of the pBend2 vector (Kim *et al.*, 1989) and analysed the migration of the circularly permuted fragments on 5 and 8% polyacrylamide gels (Fig. 5). The *PpapR* DNA fragment was used as a negative control because *in silico* modelling revealed no predicted curvature. When electrophoresed in 8% polyacrylamide gels, the toxin gene promoters exhibited an overall retarded migration that was not observed in the migration pattern of the *papR* promoter (Fig. 5a). While the 301 bp *papR* promoter fragment migrated consistently with the 300 bp marker, the 301 bp *pagAR* promoter fragment migrated to

a position well above the 300 bp marker. The slowest-migrating *pagAR* promoter fragments were those generated using *BglII* and *NheI*, which place the *pagAR* promoter insert at the end of the digested fragment (Fig. 4). The fastest-migrating fragment, and the only one with an apparent size below 400 bp, was the fragment resulting from *SpeI* digestion. In this fragment the *pagAR* promoter insert is close to the centre. Surprisingly, the fragment generated with *XhoI*, which positions the *pagAR* promoter 6 bp closer to the centre compared with the *SpeI* digest, migrates much more slowly. These results were reproduced in multiple independent experiments, including a trial in which the enzyme concentration was reduced 10-fold. The digests yielded a similar migration pattern following electrophoresis on 5% polyacrylamide gels at 4 °C (Fig. 5b). To verify that the *SpeI*-digested fragment was

Fig. 4. Schematic to indicate the positioning of predicted bends within the toxin gene promoter fragments used in circular permutation assays. The minimal functional promoter regions determined for *pagAR*, *lef* and *cya*, and the *papR* promoter regions, were cloned into the *XbaI*–*SalI* sites of vector pBend2 (Kim *et al.*, 1989). Circularly permuted promoter fragments were cleaved with restriction enzymes, which changed the positioning of the predicted bend within the same length of the DNA sequence. The schematics indicate the location of each promoter insert in the corresponding digests: 1, *BglII*; 2, *NheI*; 3, *SpeI*; 4, *XhoI*; 5, *EcoRV*; 6, *PvuII*; 7, *BamHI*. Black boxes indicate approximate locations of predicted high curvature; grey boxes indicate other regions of the promoter.

not shorter due to star activity of the *SpeI* enzyme, digestions were repeated, and the *SpeI* fragment was electrophoresed on a 2 % agarose gel, purified and subjected to PCR with pBend2-specific primers. A PCR product was generated, indicating the integrity of the digested fragment (data not shown).

The aberrant migration patterns of *lef* and *cya* promoters in 8 % polyacrylamide gels were subtle, compared with the pronounced overall retardation of the *pagAR* promoter. Yet pronounced differences in fragment migration were observed when we performed the circular permutation assays in 5 % polyacrylamide gels electrophoresed at 4 °C. In experiments shown in Fig. 5(b), the pBend2 constructs containing the *lef* and *cya* promoters were digested with *Bgl*II, *EcoRV* and *Bam*HI, placing the promoter inserts at the far right, centre, and far left of the fragments depicted in Fig. 4, respectively.

The *cya* promoter fragment (270 bp) migrated at rates similar to or slower than that of the 300 bp size marker (Fig. 5a), indicating the presence of curvature. According to our modelling results, the bends preceding the *cya* transcription start site are found between –90 and –28 bp, a region found roughly in the centre of the 137 bp promoter fragment cloned in pBend2. As expected, when this fragment was digested with *Bgl*II or *EcoRV*, both of which place the predicted bend close to the centre of the digested fragment (Fig. 4), the fragments migrated with an apparent size of 300 bp (Fig. 5b). Digestion with *Bam*HI, which places the predicted area of curvature at the extreme left of the digested fragment, resulted in faster migration and the digested sequence showed an apparent size of 275 bp (Fig. 5b).

The *lef* promoter fragment (284 bp) had an overall faster migration on 5 and 8 % polyacrylamide gels, running with an apparent size closer to 250 bp (Fig. 5), but differences in mobility of the digested *lef* pBend constructs were observed on the high-percentage polyacrylamide gels. Based on our *in silico* models, the area of high curvature within the *lef* promoter region is predicted to lie ~20 bp upstream of the *lef* transcription start and occupies ~30 bp (Fig. 4). On 5 % gels (Fig. 5b), significant differences in the mobility of the digested *lef* pBend constructs were not readily apparent. Yet 8 % gels (Fig. 5a) showed that while the *lef* pBend2 construct digested with *Bgl*II, which places the predicted bend close to the centre, ran with an apparent size of 250 bp, digestion with *EcoRV* and *Bam*HI, which shifts the location of the predicted bend away from the centre and to the extreme left, increased the rate of migration of the digested *lef* fragment to an apparent size close to 225 bp. It is important to note that for all promoters tested, the migration patterns of all digested fragments corresponded to actual fragment size, when electrophoresed on 2 % agarose gels in 1 × TBE. Similar results have been reported for other curved promoters, including the *put* control region of *S. typhimurium* (Ostrovsky de Spicer *et al.*, 1991).

(a)

DISCUSSION

The *B. anthracis* gene regulator AtxA, which tightly controls transcription of the toxin genes, does not exhibit DNA-binding specificity *in vitro*, and no *cis*-acting elements associated with AtxA-mediated regulation have been identified. The lack of information about *cis*-acting control regions of the toxin gene promoters prompted us to characterize the promoter DNA sequences. Our deletion analysis studies identified minimal sequences 5' of the toxin gene transcription start sites required for maximal and AtxA-dependent promoter activity. Given that we found no DNA sequence similarities among these coordinately regulated promoters, we postulated that they contained intrinsic structural similarity. Intrinsically

Fig. 5. Electrophoresis of the circularly permuted assay promoters. (a) Circularly permuted promoter fragments were electrophoresed in 8% (75:1) polyacrylamide gels at room temperature. (b) Circularly permuted promoter fragments were electrophoresed in 5% (29:1) polyacrylamide gels at 4 °C. Lanes: 1, *Bgl*II; 2, *Nhe*I; 3, *Spe*I; 4, *Xho*I; 5, *Eco*RV; 6, *Pvu*II; 7, *Bam*HI; S, 50 bp Step Ladder (Promega).

curved DNA sequences are usually composed of phased A- or T-tracts (Calladine *et al.*, 1988; Hirota & Ohyama, 1995). The minimal functional *pagAR*, *cya* and *lef* promoter regions determined in this study are 85, 79 and 80 % A+T, respectively: percentages significantly higher than the overall 64.6 % A+T content of the *B. anthracis* genome (Read *et al.*, 2003). The retarded migration patterns obtained in our electrophoretic mobility analyses indicate significant curvature, exceeding that predicted based on A+T content alone (Calladine *et al.*, 1988; Hirota & Ohyama, 1995).

Our modelling and circular permutation assays revealed that the most highly expressed toxin gene promoter, the *pagAR* promoter (Cataldi *et al.*, 1992), is characterized by the most pronounced curvature. *In silico* models predict that the *pagAR* promoter contains two bends oriented perpendicularly. The first bend is predicted to begin 58 bp upstream of the AtxA-mediated transcription start and occupy a 48 bp region, terminating 10 bp upstream of the start. The second bend is predicted to be ~30 bp downstream of the transcription start, terminating at the end of the sequence. The presence and organization of the two bends in the *pagAR* promoter is further supported by aberrant migration of the different *pagAR* circular

permutations in electrophoretic mobility analyses. *In silico* models generated for the *cya* and *lef* promoters suggest that these DNA sequences have a similar 3D structure (see Fig. 3, third row). In the case of the *cya* promoter DNA, the area with pronounced curvature is found the furthest upstream with respect to the *atxA*-regulated transcriptional start site, at approximately -100 bp. Interestingly, a 5'-deletion fragment spanning 101 bp upstream of the *cya* transcriptional start site was not functional. The area of high curvature predicted for the *lef* promoter region lies ~40–50 bp upstream of the transcriptional start, at a distance similar to that found in the *pagAR* promoter. Neither the *lef* or *cya* promoter regions are characterized by additional areas of high curvature. This was also reflected in the migration patterns observed in our circular permutation analyses.

In addition to at least one area of high curvature present in the three toxin promoter regions, our modelling analyses predict the presence of a distinct kink in close proximity to the *atxA*-regulated transcription start sites. In the case of the *pagAR* and *lef* promoter regions, the predicted kink in the DNA is found in very close proximity to the start sites, indicating a significant similarity between the two regions, in addition to the

location of the area of high curvature. Notably, in limited 3'-deletion analyses not shown here, we observed that ~80 bp of sequence downstream of the transcriptional start sites for *pagAR* and *lef* was required for wild-type promoter activity. On the other hand, in the *cya* promoter region, in which the kink is predicted to be farther upstream of the transcriptional start site, only 15 bp of sequence downstream of the transcriptional start site, in addition to 122 bp upstream, was enough to produce wild-type levels of activity. These observations suggest that the kinked region may be of importance to the activity of these promoters. The kinked regions identified in the toxin gene promoters have no apparent sequence similarities. Future work will include further characterization of the positioning and sequence composition of these regions.

It is notable that *B. anthracis* toxin gene expression is enhanced during growth of the bacterium in elevated atmospheric CO₂ or dissolved bicarbonate (Sirard *et al.*, 1994). It is possible that alterations in the inherent DNA topology of the *B. anthracis* toxin gene promoters occur in response to growth under these conditions. Local DNA structure can be affected by environmental conditions. DNA topology changes due to environmental cues, including osmolarity (Jordi *et al.*, 1995), temperature (Goldstein & Drlica, 1984) and oxygen availability (Dorman *et al.*, 1988), have been reported. For example, a DNA topological change required for transcription from the *proU* promoter in *S. typhimurium* is induced under conditions of high osmolarity (Jordi *et al.*, 1995; Owen-Hughes *et al.*, 1992; Yamada *et al.*, 1991; Zuber *et al.*, 1994). In the same organism, DNA topology of the *aniG* promoter is affected by changes in pH (Karem & Foster, 1993).

Overall, our analysis of the promoter regions of the anthrax toxin promoters has revealed the minimal regions required for promoter activity, and areas of high curvature within these sequences. The lack of any known sigma factor consensus sequences may indicate that in the absence of AtxA these plasmid-borne promoter regions are not favoured for expression by the conventional transcription machinery. The presence of high intrinsic curvature may play a role in recognition of the promoters by transcription machinery and/or *trans*-acting regulators such as AtxA. Structural models of our non-functional deleted promoters predict alterations in the intrinsic curvature, suggesting that the architecture of the DNA within these regions plays a significant part in toxin gene expression. We postulate that in an organism with a high A + T content, such robust intrinsic curvature may preclude the need for nucleosome architects such as H-NS and HU. Our study is, to our knowledge, the first comprehensive analysis of the toxin gene promoter regions and *cis*-acting sequences important for expression. These findings will complement ongoing investigations of AtxA and other *trans*-acting factors that affect promoter expression.

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