The DNA-binding specificity of the Bacillus anthracis AbrB protein

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The Bacillus subtilis AbrB protein is a DNA-binding global regulator of a plethora of functions that are expressed during the transition from exponential growth to stationary phase and under suboptimal growth conditions. AbrB orthologues have been identified in a variety of prokaryotic organisms, notably in all species of Bacillus, Clostridium and Listeria that have been examined. Based on amino acid sequence identity in the N-terminal domains of the orthologues from B. subtilis and Bacillus anthracis, it was predicted that the proteins might display identical DNA-binding specificities. The binding of purified B. anthracis AbrB (AbrBBA) and purified B. subtilis AbrB (AbrBBs) at DNA targets of B. subtilis, B. anthracis and a synthetic origin was compared. In all cases examined, DNA-binding specificity was identical as judged by DNase I footprinting. In B. subtilis cells, the B. anthracis promoters from the atrA and abrB genes were regulated by AbrBBs, and the B. subtilis promoter from the yxbB operon was regulated by AbrBBA.

INTRODUCTION

One of the most frequently encountered large-scale stresses that a cell faces is nutrient limitation. Upon depletion of one or more vital nutrients, Bacillus subtilis enters a transition state of unbalanced growth preparatory to either its entry into a semi-quiescent stationary phase of low metabolic activity or progression into a developmental pathway leading to endospore formation. The AbrB protein is a pivotal regulator of transition-state gene expression serving to integrate metabolic and environmental information, and channel the cell along the proper path while ensuring that metabolites and energy sources necessary for the task are readily available (Phillips & Strauch, 2002; Strauch, 1993; Strauch & Hoch, 1993). The size and diversity of functions present in the AbrB regulon indicate the global role of AbrB in optimizing gene expression to suit a particular environment (Phillips & Strauch, 2002; Strauch, 1993). AbrB orthologues and paralogues have been found in all Bacillus, Clostridium and Listeria species sequenced to date. Others have been noted via BLAST (Altschul et al., 1990) searches in Oceanobacillus, Geobacillus, Ruminococcus, Thermoanaerobacter, Moorella, Desulfitobacterium, Exiguobacterium, Carboxydothermus, Pyrococcus, Sulfolobus and Ferroplasma databases.

The B. subtilis AbrB protein is a homotetramer of identical 10 500 Da subunits (Vaughn et al., 2000; Perego et al., 1988). The N-terminal domain (about 50 amino acids) of the protein is paradigmatic for a new class of DNA-binding motif (the looped-hinge helix fold) that achieves binding specificity through flexible interactions with DNA of varying sequences (Vaughn et al., 2000; Zuber, 2000). Based upon mutant analysis (Xu et al., 1996) and examination of purified N-terminal truncations of AbrB (Xu & Strauch, 2001), it has been proposed that most, if not all, of the DNA-binding specificity determinants reside in the N-terminal domain, with the function of the C-terminal being a multimerization domain. The AbrB protein from Bacillus anthracis is highly homologous to the B. subtilis protein (80/94 amino acid residue identities), with the N-terminal 62 residues being perfectly identical in the two species. Given this identity in DNA-binding domains, and our hypothesis that the C-terminal domains of AbrB homologues are primarily multimerization domains, we predicted that the proteins might be interchangeable in achieving AbrB-mediated regulatory effects in vivo, and that the sequence differences in the C-terminal domains of the proteins might have little, or no, effect on DNA-binding specificity. To test these predictions, we purified the B. anthracis AbrB protein (AbrBB) and compared its in vitro binding specificity to that of the B. subtilis orthologue (AbrBBs). Additionally, we examined the ability of the two orthologues to substitute for each other in achieving in vivo regulatory effects.

METHODS

Expression and purification of AbrB proteins. B. subtilis AbrB was expressed in Escherichia coli and purified as has been described
(Strauch et al., 1989b), with the following modifications: buffer B* (see below) replaced the buffer A of the original purification scheme, and the desalting step prior to fractionation on DEAE-trisacryl column was accomplished by dialysis rather than use of a desalting column. A 450 bp DNA fragment containing the B. anthracis abrB gene and its ribosome-binding site was amplified via PCR using pUTE460 (T. Koehler, University of Texas, Houston, TX, USA) as a template, and the oligonucleotide primers S95 (5′-CGGAAATTCGTGCATAGGTATTGTTGCG-3′) and S96 (5′-AAATCTCGAGGATAGTTGCGAAGC-3′). After digestion with EcoRI and PstI (which cut at sites engineered at the flanks; see the underlining in the primers above), the fragment was ligated into the IPTG-inducible expression vector pQKV4 (Strauch et al., 1989b), creating plasmid pMAS3317, which was introduced into E. coli strain DH5α. The resultant expression strain (MAS3317) was grown at 37 °C in LB medium containing 50 μg ampicillin ml⁻¹ to an OD₆₀₀ of 0.6. IPTG was added to 1 mM and incubation continued for 2 h. The cells were harvested by centrifugation, washed and cracked open as has been described (Strauch et al., 1989b). Throughout the procedure the presence of the AbrBBS protein was monitored by SDS-polyacrylamide gel fractionation (4% stacking gel/18% separating gel; Laemmli buffer system). All the following steps were performed at 4 °C. The buffer (B*) used to prepare the crude extract and in the following steps was 10 mM Tris pH 8, 10 mM KCl, 0-1 mM MgCl₂, 1 mM Na₂EDTA, 10 mM β-mercaptoethanol and 40 μg PMSF ml⁻¹. The crude extract was subjected to streptomycin sulfate and ammonium sulfate precipitation steps as has been described for the B. subtilis protein, except that the ammonium sulfate cut used for subsequent steps was 30-75%. After dialysis versus buffer B*, the (NH₄)₂SO₄ cut was applied to a DEAE-trisacryl (BioSep) column, washed with buffer B* and eluted with a 10-400 mM gradient of KCl in buffer B#. Fractions containing the AbrBBS protein were pooled, dialysed versus buffer C* (25 mM Tris pH 7-2, 5 mM KCl, 1 mM MgCl₂, 1 mM Na₂EDTA, 10 mM β-mercaptoethanol and 40 μg PMSF ml⁻¹) and applied to a heparin agarose (Sigma) column equilibrated with the same buffer. The column was washed with buffer C* and eluted with a 5-150 mM KCl gradient in C*. Fractions containing the AbrBBA protein were pooled and concentrated to approximately 2-3 mg ml⁻¹ using Centriprep 3 devices (Amicon). Glycerol was added to approximately 25% (v/v; final concentration) for storage at −80 °C. Protein concentrations were determined using the Bio-Rad (Bradford) assay reagent with BSA as the standard. Final protein purity was judged to be greater than 95% by SDS-PAGE (data not shown).

Subcloning of B. anthracis promoters for in vitro and in vivo assays. A 372 bp fragment containing the attA promoter region (−200 to +171, relative to the start of transcription) was PCR amplified using pUTE411 as the template and the following primers: 5′-GGGAATTCG-TAACTACTTATCATATACAGTGACACAT-3′ and 5′-TTGCGGATCCGTGATACCATGAAA-171 relative to the ATG start codon of the pag gene) was amplified using pUTE5 as the template and the primers 5′-CGGAAATTCGAGCTCAGATATGTTGCGAAGC-3′ and 5′-ATGGATCCCGGCATTGCAGATTTGGCGA-3′. A 400 bp fragment containing the presumptive cya promoter region (−317 to +83 relative to the ATG start codon of the cya gene) was amplified using pUTE3 as the template and the primers 5′-CGGAAATTCGAGCTCAGATATGTTGCGAAGC-3′ and 5′-ATGGATCCCGGCATTGCAGATTTGGCGA-3′. A 326 bp fragment containing the presumptive promoter region of the pag gene (−230 to +96 relative to the ATG start codon of the pag gene) was amplified using pUTE4 as the template and the primers 5′-CGGAAATTCGAGCTCAGATATGTTGCGAAGC-3′ and 5′-ATGGATCCCGGCATTGCAGATTTGGCGA-3′. The fragments were cloned into either pDH32, a lacZ transcriptional fusion vector integrating at the amyE gene of B. subtilis (Shimosu & Henner, 1986), or pM103 (Perego, 1993), or both. The appropriate B. subtilis strains were transformed with the pDH32 derivatives carrying the attA and abrBpromoters using standard procedures (Hoch, 1991), and were assayed for β-galactosidase activity as described below. Derivatives of pM103 were used to generate labelled fragments for use in footprinting experiments (see below).

Construction of reporter strains SQQ103 and SQQ118. An approximately 450 bp fragment containing the ycbB promoter (Yoshida et al., 1995, 1999; Molle et al., 2004) was inserted into the lacZ fusion vector pDG1729 (Guerout-Fleury et al., 1996; pDG1729 is an integrative plasmid that inserts in the tbr locus of B. subtilis) to create pQQ23. The spo0a abrB strain S111 (Xu et al., 1996) was transformed with pQQ23, with selection for spectinomycin resistance (75 μg ml⁻¹) and screening for threonine auxotrophy, to produce strain SQQ107. DNA fragments (about 450 bp) containing the abrB genes from B. subtilis and B. anthracis, and their respective ribosome-binding sequences, were inserted into the amyE-integrative vector pDR67 (Ireten et al., 1993) in order to place the genes under control of the IPTG-inducible psAP promotor present on pDR67. These latter plasmids, pQQ43 and pQQ47, were propagated in a pcn mutant E. coli strain (TP611) as it had been determined empirically that psAP derivative plasmids expressing wild-type alleles of abrB were deleterious to E. coli cells. Plasmids pQQ43 and pQQ47 were introduced into SQQ107, with selection for chloramphenicol resistance (5 μg ml⁻¹) and screening for the absence of amylose activity (on nutrient agar plates containing cornstarch), to create SQQ103 and SQQ118, respectively.

DNase I footprinting assays. Target DNA fragments containing promoter regions were obtained from the appropriate clones (see above, Table 1 and figures), and end-labelled using [α-³²P]dATP (Amersham) and the Klenow enzyme (New England Biolabs). DNase I protection assays were performed essentially as has been previously described (Strauch et al., 1989b; Xu & Strauch, 2001), but with the following modifications: AbBr binding was carried out in a 15 μl reaction volume at room temperature for 10 min followed by Dnase I (6-3 μg ml⁻¹ final concentration) treatment for 10 s at room temperature.

β-Galactosidase assays. Cultures for assays of β-galactosidase activity were grown in Schaeffer’s medium and the assays were performed as has been described previously (Ferrari et al., 1986). All assays were repeated at least twice with no significant differences in either the temporal pattern or the relative levels of expression (between strains) being observed.

RESULTS AND DISCUSSION

AbrBBS and AbrBBA display identical binding specificity in vitro

The abrB gene from B. anthracis was cloned into expression
Table 1. Plasmids and parental B. subtilis strains

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>Description or use</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pDG1729</td>
<td>thrC integrative vector for lacZ fusions</td>
<td>Guerout-Fleury et al. (1996)</td>
</tr>
<tr>
<td>pDH32</td>
<td>amyE integrative vector for lacZ fusions</td>
<td>Shimotsu &amp; Henner (1986)</td>
</tr>
<tr>
<td>pDR67</td>
<td>amyE integrative vector for pSpac fusions</td>
<td>Iretion et al. (1993)</td>
</tr>
<tr>
<td>pJM103</td>
<td>cloning of PCR fragments</td>
<td>Perego (1993)</td>
</tr>
<tr>
<td>pJM5134</td>
<td>pJM103+abrbRS promoter region</td>
<td>Perego et al. (1988)</td>
</tr>
<tr>
<td>pKQV4</td>
<td>expression vector</td>
<td>Strauch et al. (1989b)</td>
</tr>
<tr>
<td>pMAS3011</td>
<td>pSE280+Abbr-binding site from spo0E RS</td>
<td>Strauch (1995a)</td>
</tr>
<tr>
<td>pMAS3317</td>
<td>pKQV4+abrbBA gene</td>
<td>This study</td>
</tr>
<tr>
<td>pMAS3340</td>
<td>pJM103+ataX A promoter</td>
<td>This study</td>
</tr>
<tr>
<td>pMAS3353</td>
<td>pDH32+abrbBA promoter (intact)</td>
<td>This study</td>
</tr>
<tr>
<td>pQQ23</td>
<td>PDG1729+yxbB–lacZ fusion</td>
<td>This study</td>
</tr>
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<td>pQQ43</td>
<td>pDR67+pSpac–abrbRS fusion</td>
<td>This study</td>
</tr>
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<td>pQQ47</td>
<td>pDR67+pSpac–abrbBA fusion</td>
<td>This study</td>
</tr>
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<td>pUITE2</td>
<td>PBSIIKS+1.5 kb fragment with ef promoter</td>
<td>T. Koehler†</td>
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<td>pUITE4</td>
<td>PBSIIKS+0.9 kb fragment with pag promoter</td>
<td>T. Koehler</td>
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<td>pUITE5</td>
<td>PBSIIKS+1.3 kb fragment with cya promoter</td>
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<td>pUITE411</td>
<td>pHT304–18Z+ataX–lacZ fusion</td>
<td>T. Koehler</td>
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<td>pUITE415</td>
<td>pGEM-T easy+abrbBA promoter region</td>
<td>T. Koehler</td>
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<tr>
<td>pUITE460</td>
<td>pGEM-T easy+abrbBA coding region</td>
<td>T. Koehler</td>
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<td>1S11</td>
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<td>BGSC; Xu et al. (1996)</td>
</tr>
<tr>
<td>JH642</td>
<td>trpC2 pheA1</td>
<td>J. Hoch§</td>
</tr>
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<td>trpC2 pheA1 spo0A204</td>
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<td>SWV217</td>
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<td>Strauch (1995b)</td>
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<td>SWV1117</td>
<td>trpC2 pheA1 spo0H81 abrb::tet</td>
<td>This study</td>
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</table>

*For brevity, most pDH32 and pJM103 derivatives of various B. anthracis promoter regions are not listed here but their construction is detailed in the text. Also for brevity, the various B. subtilis strains containing the pDH32 derivatives are not enumerated here, but their nature is given in the relevant figure legends.

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vector pKQV4 and the protein overexpressed in E. coli as described in Methods. Although identical in amino acid sequence over the first 62 residues, the B. subtilis and B. anthracis AbrB proteins are non-identical at 14 positions in their last 32 residues. These differences result in the intact AbrBBA protein being significantly more acidic than AbrBBS (calculated pI of 5.0 versus 6.3, respectively) and presumably account for the differences in behaviour of the two proteins seen during purification (different (NH4)2SO4 fractionation patterns, different affinity for binding DEAE at pH 8 and lower pH required for binding of AbrBBA to heparin agarose). We had previously shown that the majority, if not all, of the DNA-binding specificity determinants for AbrBBS reside in the N-terminal 50–55 amino acid residues (Xu & Strauch, 2001), and have hypothesized that the C-terminal residues are required primarily for stable multimerization of the native homotetramer and provide no direct DNA-binding specificity determinants (Xu et al., 1996; Xu & Strauch, 2001). Given the identical nature of the N-domains and the significantly different C-domains of AbrBBS and AbrBBA, comparison of the DNA-binding specificity of these two orthologues provided a strong test for our hypotheses.

We first performed DNase I footprinting assays of AbrBBA and AbrBBS binding to the promoter region of the abrbRS gene. As shown in Fig. 1, the extent of binding by the two proteins is indistinguishable at this target. Reasoning that expression of the B. anthracis AbrB is probably subject to autoregulation as is the B. subtilis protein (also see below), we next examined binding of the proteins to a DNA fragment containing the abrbBA promoter region. Fig. 2 shows the result of a DNase I footprinting experiment demonstrating that the extent of binding by the two proteins is indistinguishable at this target also. Fig. 3 provides a comparison of the sequences of the two promoters and...
shows the extent of AbrB binding to each. Previous work had divided the AbrB-binding region on the \textit{abrB} BS promoter into a higher-affinity interaction region (\(12\) to \(43\)) and a contiguous upstream region (\(44\) to about \(120\)) that displayed an apparently lower affinity for binding (Strauch \textit{et al.}, 1989b; Xu \textit{et al.}, 1996; Strauch, 1995a). The position (relative to RNA polymerase recognition elements) of AbrB binding on the \textit{abrB} BA promoter coincides with the higher-affinity region on the \textit{abrB} BS target (although the former extends about one turn of the helix farther downstream than the latter). In contrast, the \textit{B. anthracis} abrB promoter lacks the upstream lower-affinity region, possibly due to the upstream presence of the sequence \(5'-\text{TTTTAAAA}-3'\) (from \(52\) to \(61\)), which coincides with the higher-affinity region on the \textit{abrB} BS target (although the former extends about one turn of the helix farther downstream than the latter). In contrast, the \textit{B. anthracis} abrB promoter lacks the upstream lower-affinity region, possibly due to the upstream presence of the sequence \(5'-\text{TTTTAAAA}-3'\) (from \(52\) to \(61\)), which

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**Fig. 1.** DNase I footprinting analysis of binding to the \textit{B. subtilis} abrB promoter. The results shown are those when the non-template strand of the \textit{B. subtilis} abrB promoter region was labelled at its 3' end. Lanes 1–4, 20, 10, 4 and 0 \(\mu\)M \textit{B. subtilis} AbrB, respectively; lanes 5–8, 24, 12, 4-8 and 0 \(\mu\)M \textit{B. anthracis} AbrB, respectively. Maxam–Gilbert purine (R) and pyrimidine (Y) sequencing ladders were included for reference. Two positions relative to the start point of transcription from the P2 promoter (Perego \textit{et al.}, 1988) are indicated for orientation. The source of the labelled DNA fragment was pJM5134 (Perego \textit{et al.}, 1988).

**Fig. 2.** DNase I footprinting analysis of binding to the \textit{B. anthracis} abrB promoter. The results shown are those when the non-template strand of the \textit{B. anthracis} abrB promoter region was labelled at its 3' end. The reactions for lanes 1–8 were performed at pH 8, while those for lanes 9–11 were performed at pH 7. Lanes 1–3, 20, 10 and 4 \(\mu\)M \textit{B. subtilis} AbrB respectively; lanes 5–7, 24, 12 and 4-8 \(\mu\)M \textit{B. anthracis} AbrB respectively; lanes 4, 8 and 11, no AbrB; lane 9, 20 \(\mu\)M \textit{B. subtilis} AbrB; lane 10: 24 \(\mu\)M \textit{B. anthracis} AbrB. Maxam–Gilbert purine (R) and pyrimidine (Y) sequencing ladders were included for reference. The source of the labelled DNA fragment was pMAS3353.

**Fig. 3.** Comparison of the sequences and AbrB footprint regions in the \textit{B. subtilis} and \textit{B. anthracis} abrB promoters. The abrB promoter regions from the two species are shown in alignment, with consensus positions indicated by asterisks. The –35 and –10 regions are highlighted, as are the transcriptional start sites (+1). The regions protected from DNase I cleavage due to AbrB (either orthologue) binding are indicated by the dashed lines above (for the \textit{B. anthracis} promoter) and below (for the \textit{B. subtilis} promoter) the sequences, upstream from the +1 position. The dashed line downstream from the +1 position denotes the sequences corresponding to the Spo0A-binding recognition sites (Spo0A boxes).
is not found in the \textit{abrB}^{BS} sequence. The physiological or regulatory relevance of the upstream TTTTTAAAAA sequence, and the lack of upstream AbrB binding, is not clearly understood but \textit{in vitro} binding of the two AbrB orthologues was identical, supporting the hypothesis that sequence specificity results from interaction of the identical N-domains of the proteins with the DNA targets.

Further confirmation of the identical \textit{in vitro} binding specificity of the two AbrB proteins is shown in Fig. 4, which depicts binding to DNA fragments containing two synthetic sites selected for high-affinity binding by AbrB^{BS} (Xu & Strauch, 1996). Identical specificity (data not shown) was also seen at the AbrB^{BS} targets from the \textit{B. subtilis} yxbB and spo0E (Strauch, 1995a) genes.

**Examination of AbrB binding to promoter regions of \textit{B. anthracis} toxin genes**

Previous reports have implicated AbrB as playing a role in either the level or the timing of anthrax toxin expression (Saile & Koehler, 2002; Baillie \textit{et al}, 1998). We examined the ability of the AbrB proteins to interact with the putative promoter regions of the \textit{B. anthracis} toxin component genes (\textit{cya}, \textit{lef} and \textit{pag}) and to the promoter for the toxin regulatory gene, atxA (Dai \textit{et al}, 1995). No binding was observed to a fragment containing positions $-230$ to $+96$ (relative to the ATG start codon) of the \textit{pag} gene or to a fragment containing positions $-317$ to $+83$ (relative to the ATG start codon) of the \textit{cya} gene (data not shown). A rather weak AbrB binding interaction (data not shown) was observed upstream of the \textit{lef} gene at positions $-260$ to $-232$ relative to the mapped transcription start site (Dai \textit{et al}, 1995). As this site is well upstream of the \textit{lef} promoter, its direct regulatory significance for lethal factor expression seems doubtful. A much stronger AbrB–DNA interaction (Fig. 5) was observed in the vicinity of the promoter for \textit{atxA} expression (Dai \textit{et al}, 1995). Relative to the start point of transcription, AbrB protected a region extending from $-67$ to about $-25$ on the template strand ($-67$ to $-18$ on the non-template strand; see Fig. 6). Given this location that overlaps with crucial promoter elements, and a previous report indicating that AbrB negatively regulates \textit{atxA} expression (Saile & Koehler, 2002), it seems logical to conclude that AbrB binding here has a direct regulatory effect \textit{in vivo}.

![Fig. 4. DNase I footprinting analysis of binding to two synthetic target sites selected for high-affinity binding by AbrB^{BS}.](a) BS18 target aptamer (Xu & Strauch, 1996). Lanes 2 and 3, 2 to 4 and 12 \textit{mM} AbrB^{RA}, respectively; lanes 5 and 6, 2 and 10 \textit{mM} AbrB^{BS}, respectively; lanes 1 and 4, no AbrB. (b) C47 target aptomer (Xu & Strauch, 1996). Lanes 1 and 2, 10 and 2 \textit{mM} AbrB^{BS}, respectively; lanes 3 and 4, 12 and 2.4 \textit{mM} AbrB^{BA}, respectively; lane 5, no AbrB.

![Fig. 5. DNase I footprinting analysis of binding to the \textit{B. anthracis} atxA promoter region.](b) The results shown are those when the non-template strand of the \textit{B. anthracis} atxA promoter region was labelled at its 3’ end. Lanes 1–4, 20, 10, 4 and 0 \textit{nM} \textit{B. subtilis} AbrB, respectively; lanes 5–8, 24, 12, 4.8 and 0 \textit{nM} \textit{B. anthracis} AbrB, respectively. Maxam–Gilbert purine (R) and pyrimidine (Y) sequencing ladders were included for reference. The location of the sequences bound on both strands is shown in Fig. 6.
**AbrB\textsubscript{BS} regulates atxA–lacZ and abrB\textsuperscript{BA}–lacZ fusions in B. subtilis**

Given the identical DNA-binding specificity we observed *in vitro*, we predicted that AbrB\textsubscript{BS} would regulate the *B. anthracis* atxA and abrB promoters in *B. subtilis* cells (assuming the promoters were recognized by *B. subtilis* RNA polymerase). To determine if the atxA promoter region would be expressed in *B. subtilis* and regulated by AbrB\textsubscript{BS}, we examined expression of a lacZ transcriptional fusion to the atxA promoter in wild-type, *abrB*, spo0A, spo0H, spo0A abrB and spo0H abrB strains of *B. subtilis*. As shown in Fig. 7, the atxA promoter is expressed in *B. subtilis* and subject to AbrB-dependent regulation. Expression was significantly increased in an abrB mutant strain (relative to wild-type) during both vegetative growth and the early stages of stationary phase/sporulation. The decreased expression seen in spo0A or spo0H backgrounds was abrogated if an abrB mutation was also present in the strain, and the pattern and level of expression seen in the double mutants were indistinguishable from those seen in the abrB mutant. Thus the spo0A and spo0H effects appear to be solely due to their causing an increase in intracellular levels of AbrB (Strauch *et al.*, 1990).

Transcription from the abrB\textsuperscript{BA} promoter was also seen to be regulated by AbrB\textsubscript{BS} in *B. subtilis* cells. When the promoter contained its intact region for Spo0A binding (tandem Spo0A-boxes located from +11 to +27 downstream of the start site), expression in both wild-type and a spo0A mutant strain was not significantly detectable above background, but significant expression was seen in abrB and spo0A abrB strains (Fig. 8). The negligible expression of the abrB\textsuperscript{BA} promoter in wild-type and spo0A contrasts with the pattern seen for the *B. subtilis* abrB promoter in that expression of the latter is detectable in both these backgrounds, with expression in spo0A actually being slightly higher than wild-type (Strauch *et al.*, 1989a). We suspected that our observed results with the abrB\textsuperscript{BA} promoter might have been due to it being more sensitive to AbrB-mediated negative regulation than the abrB\textsubscript{BS} promoter.

Since transcription of abrB\textsuperscript{BA} is subject to repression by the Spo0A protein (Saile & Koehler, 2002), we next examined the expression of an abrB\textsuperscript{BA} variant promoter that did not possess an intact Spo0A-box region (see Methods) in order to eliminate the direct Spo0A-binding component from our analysis. Expression of the truncated construct was significant in a wild-type background, negligible in a spo0A background and greatly overexpressed (relative to the wild-type) in both abrB and spo0A abrB backgrounds (Fig. 9). The simplest explanation of these patterns is that overexpression of AbrB\textsubscript{BS} (resulting from

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**Fig. 6.** Sequence protected by AbrB in the atxA promoter. The lines denote the sequences protected from DNase I digestion due to AbrB (either orthologue) binding. The $-35$ (5’-TTCCCA-3’) and $-10$ (5’-TATAAT-3’) regions of the atxA promoter are shown in bold.

**Fig. 7.** AbrB regulation of atxA promoter expression in *B. subtilis* cells. The time-course shows β-galactosidase accumulation from the atxA′–lacZ fusion in the following *B. subtilis* genetic backgrounds: wild-type (●), spo0A (◆), abrB (▲), spo0H (▼), spo0A abrB (■) and spo0H abrB (○). Zero on the abscissa denotes the approximate end of exponential growth and entry into stationary phase.

**Fig. 8.** AbrB regulation of abrB\textsuperscript{BA} promoter expression in *B. subtilis* cells. The time-course shows β-galactosidase accumulation from the abrB\textsuperscript{BA}′–lacZ fusion containing intact Spo0A-boxes in the following *B. subtilis* genetic backgrounds: wild-type (●), spo0A (◆), abrB (▲) and spo0A abrB (■). Zero on the abscissa denotes the approximate end of exponential growth and entry into stationary phase.
lack of Spo0A transcriptional repression of the native abrB gene, in a spo0A background) completely blocks transcription from the abrB\textsuperscript{BA} promoter, presumably due, at least in part, to a strong AbrBB\textsuperscript{BS}–abrB\textsuperscript{BS} DNA-binding interaction that effectively prevents RNA polymerase access. Since overexpression of AbrB\textsuperscript{BS} (due to a spo0A mutation) does not completely shut off expression of the abrB\textsuperscript{BS} promoter, with (Strauch et al., 1989a) or without intact Spo0A-boxes (data not shown), it is possible that the abrB\textsuperscript{BS} recognition site provides a stronger binding target for AbrBB\textsuperscript{BS} interaction than does the abrB\textsuperscript{BS} recognition site, at least under the B. subtilis intracellular conditions. However, we have not been able to detect any significant difference in the strength of binding of AbrB\textsuperscript{BS} to the abrB\textsuperscript{BS} or abrB\textsuperscript{BS} promoters in vitro, nor have we detected any significant difference in the binding of AbrB\textsuperscript{BA} to these regions (data not shown). Assuming AbrB binding strength is also approximately the same at both the abrB\textsuperscript{BS} and abrB\textsuperscript{BS} promoters in vivo, perhaps the RNA polymerase interaction at the B. anthracis abrB promoter is weaker than at the homologous promoter in B. subtilis. Thus AbrB binding at the former might be more effective at blocking transcription than at the latter. If this were true, the nucleotide sequence variations between the promoters would not doubt be a major contributing factor. In this regard it is interesting to note the differing -10 sequences and the stretch of upstream A and T residues in the B. anthracis promoter, which might be a region of bending or curvature (Crothers et al., 1990). Nevertheless, our observations show that the abrB\textsuperscript{BS} promoter is an in vivo binding and regulatory target for AbrBB\textsuperscript{BS}. Although work has been published that demonstrates Spo0A regulation of abrB in B. anthracis (Saile & Koehler, 2002), we are unaware of any published data directly demonstrating autoregulation of abrB expression in B. anthracis. Combined with our other observations, we predict that the B. anthracis abrB gene is autoregulated in a manner similar, or identical, to the situation in B. subtilis.

**AbrB\textsuperscript{BA} expression in B. subtilis complements a deletion of the abrB\textsuperscript{BS} gene**

We next wished to determine if the B. anthracis AbrB protein functioned in the B. subtilis intracellular environment. Into a strain (1S11) mutant for both spo0A and abrB we ectopically placed a yxbB–lacZ reporter gene at the thrC locus. (B. anthracis does not possess a homologue of the B. subtilis yxbB operon.) At the amyE locus of the resultant strain (SQQ107), we placed either a pSpac–abrB\textsuperscript{BS} or a pSpac–abrB\textsuperscript{BA} construct to create strains SQQ103 and SQQ118, respectively. Therefore, the only functional AbrB protein expressed in these reporter strains would be the result of IPTG-inducible expression of the pSpac promoters. Fig. 10 illustrates the expression of the yxbB–lacZ reporter in these strains with and without IPTG induction (i.e. with and without expression of the abrB genes). In the absence of IPTG induction, yxbB–lacZ expression patterns were identical for the two strains. In the presence of IPTG, induction of either AbrB\textsuperscript{BS} or AbrB\textsuperscript{BA} led to repression of the yxbB–lacZ reporter. The slight difference in repression level seen for the two induced strains may be attributable to a post-transcriptional event affecting AbrB protein levels. We believe the most likely cause is a difference in translational efficiency of the abrB genes since the two pSpac constructs have slightly different ribosome-binding sites upstream of the abrB genes (each being preceded by its native RBS). Nevertheless, the results clearly show that the B. anthracis AbrB protein can complement the deletion of the abrB\textsuperscript{BS} gene in a B. subtilis cellular environment.

**Fig. 9.** AbrB regulation of an abrB\textsuperscript{BA} promoter variant lacking intact Spo0A boxes. The time-course shows β-galactosidase accumulation from the abrB\textsuperscript{BA}–lacZ fusion lacking intact Spo0A-boxes in the following B. subtilis genetic backgrounds: wild-type ( ), spo0A ( ), abrB ( ) and spo0A abrB ( ). Zero on the abscissa denotes the approximate end of exponential growth and entry into stationary phase.

**Fig. 10.** AbrB\textsuperscript{BA} regulates the B. subtilis yxbB promoter. The time-course shows β-galactosidase accumulation from a yxbB\textsuperscript{BA}–lacZ fusion in strains expressing either AbrB\textsuperscript{BS} or AbrB\textsuperscript{BA} from IPTG-inducible pSpac fusion constructs. Solid symbols: no IPTG induction; open symbols: induction with 0.1 mM IPTG. •: B. subtilis AbrB under pSpac control; ■: B. anthracis AbrB under pSpac control. Zero on the abscissa denotes the approximate end of exponential growth and entry into stationary phase. IPTG was added to the indicated cultures at approximately 1 h prior to the end of exponential growth.
Conclusions

We had previously proposed that the DNA-binding specificity determinants of the B. subtilis AbrB protein reside primarily within the N-terminal domain of the monomers that associate to form the homotetrameric native form of the protein (Xu & Strauch, 2001; Xu et al., 1996; Strauch, 1996). NMR structural studies revealed a new class of DNA-binding motif (the looped-hinge helix fold) that is formed via the association of AbrB N-domains (Vaughn et al., 2000). We further hypothesized that the C-terminal domain of the protein was primarily, or solely, a higher-order multimerization domain with no function in direct contact or recognition of target binding sites. The AbrB protein from B. anthracis has perfect amino acid sequence identity to the B. subtilis protein over the first 62 residues, but differs significantly (14 non-identities) from it in the last 32 residues. This sequence difference results in the B. anthracis protein having a significantly more acidic pI than its orthologue. To test our domain hypothesis, we examined the in vitro binding specificity and in vivo regulatory interchangeability of these two AbrB variants.

Binding specificity of the two AbrB proteins was indistinguishable at each of the target DNA sequences examined (Figs 1–6). Based on in vivo assays in an identical intracellular environment (B. subtilis), the proteins appeared essentially interchangeable in their ability to regulate each of the target promoters we examined (Figs 7–10), even when the target was a promoter not found in the protein’s normal cellular environment (e.g. atxA regulation by AbrBBA and yxbB regulation by AbrBBA). Thus, we conclude that the C-domains of the B. subtilis and B. anthracis AbrB proteins, despite their differing sequences, do not contribute to the DNA-binding specificity of the proteins. This strongly supports a notion that the N-terminal and C-terminal domains of AbrB homologues are separable modules: the N-terminal domain providing the structure that directly recognizes and interacts with DNA target sequences, and the C-domain involved solely in a multimerization interaction necessary for stable association of the active multimeric form of the protein. Finally, our results have potential implications regarding regulatory phenomena controlling toxin production in B. anthracis. Previous observations (Saile & Koehler, 2002) indicated that anthrax toxin production is regulated by AbrB and that atxA expression is elevated in abrB mutants. Our results suggest that direct AbrB binding to the atxA promoter, but not direct AbrB binding to the pag, lef or cya promoters, is responsible (at least in part) for the observed AbrB effects on toxin gene expression.

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