Loss of $\sigma^I$ affects heat-shock response and virulence gene expression in *Bacillus anthracis*

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The pathogenesis of *Bacillus anthracis* depends on several virulence factors, including the anthrax toxin. Loss of the alternative sigma factor $\sigma^I$ results in a coordinate decrease in expression of all three toxin subunits. Our observations suggest that loss of $\sigma^I$ alters the activity of the master virulence regulator AtxA, but atxA transcription is unaffected by loss of $\sigma^I$. $\sigma^I$-containing RNA polymerase does not appear to directly transcribe either atxA or the toxin gene pagA. As in *Bacillus subtilis*, loss of $\sigma^I$ in *B. anthracis* results in increased sensitivity to heat shock and transcription of sigI, encoding $\sigma^I$, is induced by elevated temperature. Encoded immediately downstream of and part of a bicistronic message with sigI is an anti-sigma factor, RsgI, which controls $\sigma^I$ activity. Loss of RsgI has no direct effect on virulence gene expression.

sigI appears to be expressed from both the $\sigma^I$ and $\sigma^A$ promoters, and transcription from the $\sigma^A$ promoter is likely more significant to virulence regulation. We propose a model in which $\sigma^I$ can be induced in response to heat shock, whilst, independently, $\sigma^I$ is produced under non-heat-shock, toxin-inducing conditions to indirectly regulate virulence gene expression.

**INTRODUCTION**

*Bacillus anthracis* is a Gram-positive, endospore-forming bacterium and is the aetiologic agent of anthrax (Koehler, 2009). Spores of *B. anthracis* can cause three different modes of infection according to their method of entry: cutaneous, pulmonary and gastrointestinal (Dixon et al., 1999). Upon entry into the host, *B. anthracis* spores germinate into vegetative cells and begin to produce a number of virulence factors, including anthrax toxin and capsule (Koehler, 2009). The pathogenic damage caused by vegetative cells results in death of the host, spore formation and spore dissemination. Virulence of *B. anthracis* is conferred by two virulence plasmids: pXO1 and pXO2. The pXO1 plasmid carries genes encoding the anthrax toxin subunits: pagA, encoding protective antigen, *lef*, encoding lethal factor, and *cya*, encoding oedema factor (Koehler, 2009; Okinaka et al., 1999b). pXO1 also carries the gene encoding the master regulator of toxin gene expression, AtxA (Koehler et al., 1994; Uchida et al., 1993). The pXO2 virulence plasmid carries a five-gene operon that encodes the enzymes responsible for the synthesis of the poly-$\gamma$-$\delta$-glutamic acid capsule and two regulators of capsule production, AcpA and AcpB (Drysdale et al., 2004; Okinaka et al., 1999a; Vietri et al., 1995). Expression of virulence factors is influenced by environmental signals, such as temperature and bicarbonate levels, indicative of presence in the mammalian host (Bartkus & Leppla, 1989; Drysdale et al., 2005; Hammerstrom et al., 2011; Sirard et al., 1994).

Furthermore, virulence gene regulation is influenced by the growth state of the cell (Château et al., 2011; Chiang et al., 2011; Drysdale et al., 2005; Koehler et al., 1994; Saile & Koehler, 2002; Tsvetanova et al., 2007; Wilson et al., 2009).

In response to changing environmental conditions, many bacteria use alternative sigma factors to simultaneously control the transcription of multiple genes. Sigma factors are responsible for promoter recognition and will bind to the RNA polymerase (RNAP) core to initiate transcription at specific promoters. Many sigma factors are regulated post-translationally by anti-sigma factors that bind to the sigma factor and prevent its interaction with RNAP. The anti-sigma factor is released in response to cellular signals, freeing the sigma factor to interact with RNAP to direct transcription of target genes (Paget, 2015). Of the 27 predicted sigma factors in the *B. anthracis* genome (Schmidt et al., 2011), only a small number have been studied experimentally: $\sigma^H$ is involved in the heat-shock response and influences virulence in a mouse model of infection (Fouet et al., 2000), $\sigma^{11}$ is required for sporulation and influences toxin gene expression in some *B. anthracis* strains (Bongiorni et al., 2008; Hadjifragiskou et al., 2007), and $\sigma^P$ controls $\beta$-lactamase in a penicillin-resistant *B. anthracis* strain (Ross et al., 2009).

In *Bacillus subtilis*, $\sigma^I$, formerly known as YkoZ, is an alternative sigma factor associated with a number of cellular processes, including cell wall metabolism, cell division, nutrient

**Abbreviation:** RNAP, RNA polymerase.

One supplementary table is available with the online Supplementary Material.
regulation, antibiotic resistance and stress response (Asai et al., 2007; Huang et al., 2013; Salzberg et al., 2013; Tseng & Shaw, 2008; Tseng et al., 2011; Zuber et al., 2001). σ1 belongs to the σ70 family of sigma factors and is a member of the group VI B. subtilis heat-shock genes (Schumann, 2003; Zuber et al., 2001). Loss of σ1 reduces survival at high temperature and transcription of sigl, encoding σ1, is induced by heat (Zuber et al., 2001). Transcription of the sigl gene is initiated from both the σ1 and σA promoters (Asai et al., 2007; Huang et al., 2013; Salzberg et al., 2013). sigl expression is repressed by glucose, but this repression is not dependent upon a predicted CcpA binding site upstream of the σ1–35 promoter region. σ1 activity is controlled by the anti-σ1 factor Rsgl, and sigl and rsgl are co-transcribed as a single bicistronic mRNA initiated from the promoters upstream of sigl (Asai et al., 2007; Salzberg et al., 2013). Without Rsgl, sigl–rsgl transcription was increased in the absence of heat shock. The chaperone DnaK may also facilitate σ1 activation (Asai et al., 2007). A consensus σ1 recognition sequence was derived from mapping of sigl promoters among several Bacillus species. A bioinformatic search of the B. subtilis genome using this consensus revealed several putative σ1-controlled genes with diverse functions: bcrC (bacitracin resistance protein), lytE (cell wall hydrolase), mreBH (cell shape-determining protein), ysfD (putative glycolate oxidase subunit) and ytpS (DNA translocase) (Biller & Burkholder, 2009; Tseng & Shaw, 2008). σ1-dependent transcriptional initiation was demonstrated for the bcrC, mreBH and lytE promoters (Tseng & Shaw, 2008; Tseng et al., 2011). Loss of lytE expression is responsible for the heat-sensitive phenotype associated with loss of σ1 (Tseng et al., 2011). The WalRK (YycFG) two-component system controls sigl–rsgl expression through a WalR binding site near the σ1 promoter (Huang et al., 2013; Salzberg et al., 2013). Binding of WalR represses expression from the σ1 promoter and activates expression from the σA promoter under non-stress conditions. WalR control of sigl–rsgl and lytE expression and σ1 control of sigl–rsgl and lytE expression indicate cooperative regulation between WalR and σ1-Rsgl (Salzberg et al., 2013).

Here, we show that σ1 contributes to both stress response and virulence gene expression in B. anthracis. Loss of sigl resulted in reduced heat survival and sigl expression was induced by elevated temperature. Under non-heat-shock, toxin-inducing conditions, expression of the three toxin subunit genes was coordinately reduced in the absence of σ1. Reduced toxin gene expression was not the result of altered atxA expression and σ1–RNAP did not directly transcribe atxA or the protective antigen gene. As with B. subtilis, σ1 was regulated by the anti-sigma factor Rsgl. Together, our results suggest that σ1 indirectly regulates toxin gene expression separately from its role in heat-shock response.

METHODS

Bacterial strains and growth conditions. B. anthracis strain 34F2 (pXO1+ pXO2–) and its derivatives were routinely grown in LB or brain heart infusion (BHI) supplemented with the appropriate antibiotics according to specific plasmids used with the appropriate concentrations: chloramphenicol (7.5 μg ml–1) erythromycin (5 μg ml–1), lincomycin (25 μg ml–1) and kanamycin (7.5 μg ml–1). X-Gal (40 μg ml–1) was added to LB agar to monitor β-galactosidase activity as necessary. When indicated, B. anthracis was grown in LB-bic (LB containing 0.1 M HEPES, pH 8.0 and 0.8% NaHCO3) under 5% CO2 with agitation. Electroporation of plasmids was performed using competent cells of B. anthracis prepared as described previously (Han & Wilson, 2013; Koehler et al., 1994).

Escherichia coli TGI, C600, BL21(DE3) and DH5α competent cells were used for the propagation and isolation of all plasmid constructs. E. coli TGI, C600 and DH5α competent cells were prepared chemically as described previously (Sambrook & Russell, 2001), and transformants were selected on LB agar supplemented with ampicillin (100 μg ml–1), chloramphenicol (7.5 μg ml–1) or kanamycin (30 μg ml–1).

Plasmid and strain construction. Strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers are listed in Table S1 (available in the online Supplementary Material). Markerless gene deletion strains of BAS3231 (AW-A094), BAS3230 (AW-A118) and BAS3231–BAS3230 (AW-A125) were generated using the indicated plasmids as described previously (Bongiorni et al., 2007; Cybulski et al., 2009; Han & Wilson, 2013; Janes & Stibitz, 2006). Plasmid pAW376 was generated by transferring the pSPAC promoter from plasmid pH3155 (Wörner et al., 2006) into the EcoRI site of pORI-Cm (Bruning et al., 2005). This resulted in an integrative vector that could disrupt or restore target loci whilst still promoting transcription of downstream genes. The BAS3231 complementation strain (AW-A118) was created by integrating plasmid pAW429, carrying the upstream and coding regions of BAS3231, into the AW-A094 strain via allelic exchange, thereby restoring BAS3231 under the control of its native promoters to the BAS3231 markerless deletion strain. The retention of plasmid pXO1 in B. anthracis strains was confirmed by PCR using primers atxA U5′Bam and atxA D3′Pst (Han & Wilson, 2013).

Operon analysis. Operon organization was determined by reverse transcription (RT)-PCR using 34F2 RNA extracted using an Ultra-Clean Microbial RNA Isolation kit (MoBio). Following extraction, RNA was treated with Turbo DNase (Thermo Fisher). cDNA was synthesized using SuperScript III reverse transcriptase and random hexamers (Life Technologies). Sets of primers flanking the intergenic regions for each gene pair were used to amplify cDNA. Amplicons were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide and visualized on a UVP gel documentation system.

β-Galactosidase assays. B. anthracis strains harbouring promoter fusions on the replicative vector pTCV-lac (Poyart & Trieu-Cuot, 1997) were grown at the temperatures indicated in LB or LB-bic supplemented with the appropriate antibiotics under 5% CO2. β-Galactosidase activity was assayed as described previously and specific activity was expressed in Miller units (Miller, 1972; Wilson et al., 2008).

Heat survival assay. Survival of B. anthracis was assayed through a modification of the technique used previously (Zuber et al., 2001). Strains were struck and grown overnight at 37 °C on LB agar plates. Strains were then transferred to 6 ml LB at starting OD600 0.01 and grown at 37 or 44 °C for 18 h. Survival of strains was determined using serial dilution and colony counting after incubation at 37 °C for 15 h.

Purification of σ1. Plasmid pAW410 contained the coding sequence of B. anthracis BAS3231 (excluding the initial start codon) in the E. coli expression vector pET15b. The plasmid was constructed so as
Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tr>
<td>Strains</td>
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<tr>
<td>34F2</td>
<td>pXO1&lt;sup&gt;+&lt;/sup&gt; pXO2&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Laboratory stock</td>
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<td>AW-A094</td>
<td>Markerless deletion of &lt;i&gt;sigI&lt;/i&gt; (BAS3231)</td>
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<td>AW-A118</td>
<td>Markerless deletion of &lt;i&gt;rsgI&lt;/i&gt; (BAS3230)</td>
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<td>AW-A125</td>
<td>Markerless deletion of &lt;i&gt;sigI–rsgI&lt;/i&gt; (BAS3231–BAS3230)</td>
<td>This study</td>
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<td>AW-A136</td>
<td>Complementation of &lt;i&gt;sigI&lt;/i&gt;</td>
<td>This study</td>
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<td>Plasmids</td>
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<tr>
<td>pORI-Cm</td>
<td>Temperature-sensitive shuttle vector, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Brunsing et al. (2005)</td>
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<tr>
<td>pORI-Cm-lScel</td>
<td>pORI-Cm vector with I-SceI recognition site, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Bongiorni et al. (2007)</td>
</tr>
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<td>pSS4332</td>
<td>I-SceI expression plasmid, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Cybulski et al. (2009)</td>
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<td>pHT315s</td>
<td>Shuttle vector with P&lt;sub&gt;SPAC&lt;/sub&gt; promoter, Amp&lt;sup&gt;R&lt;/sup&gt;, Erm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Wörner et al. (2006)</td>
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<td>pAW336</td>
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<td>pAW384</td>
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<td>pAW397</td>
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<td>pAW376</td>
<td>pORI-Cm vector with P&lt;sub&gt;SPAC&lt;/sub&gt; promoter, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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<td>This study</td>
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<td>Promoterless vector for transcriptional lacZ fusion, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Poyart &amp; Trieu-Cuot (1997)</td>
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<td>&lt;i&gt;pagA&lt;/i&gt;–&lt;i&gt;lacZ&lt;/i&gt; transcriptional fusion in pTCV-lac, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tsvetanova et al. (2007)</td>
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<td>Bongiorni et al. (2008)</td>
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<td>pAW193</td>
<td>&lt;i&gt;cya&lt;/i&gt;–&lt;i&gt;lacZ&lt;/i&gt; transcriptional fusion in pTCV-lac, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<tr>
<td>pAW194</td>
<td>&lt;i&gt;lef&lt;/i&gt;–&lt;i&gt;lacZ&lt;/i&gt; transcriptional fusion in pTCV-lac, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pAW399</td>
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<td>This study</td>
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<td>pET15b</td>
<td>&lt;i&gt;E. coli&lt;/i&gt; inducible protein expression vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Laboratory stock</td>
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<td>pAW410</td>
<td>BAS3231 protein expression, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
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In <i>vitro transcription</i>. DNA templates for <i>in vitro</i> transcription were amplified by PCR, purified using an EZNA Cycle-Pure kit (Omega) and visualized by agarose gel electrophoresis using SYBR Gold (Life Technologies). The correctly sized products were then agarose gel purified using an EZNA Gel Extraction kit (Omega), quantified using a bio-spectrophotometer (Eppendorf) and visualized by agarose gel electrophoresis stained with SYBR Gold. <i>In vitro</i> transcription buffer contained 50 mM potassium acetate, 8.1 mM magnesium acetate, 50 mM Tris/acetate, pH 8.0, 27 mM ammonium acetate and 2 mM dithiothreitol (Wilson & Tan, 2002). Reactions containing <i>in vitro</i> transcription buffer, 1 pmol DNA template, 5 μg recombinant <i>B. anthracis</i> σ<sup>1</sup> and 5 U <i>E. coli</i> RNAP core (Epicon) were pre-incubated at 37 °C for 30 min. ATP, CTP, UTP and GTP were added to a final concentration of 500 μM each, and reactions incubated an additional 15 min at 37 °C. The reactions were terminated by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Samples were then electrophoresed on a 1.5% formamide agarose gel (Sambrook & Russell, 2001), stained with SYBR Gold and visualized at 495 nm on a BioDoc-It system (UVP).

**RESULTS**

Identification and description of <i>sigI</i>–<i>rsgI</i> loci

The <i>B. anthracis</i> gene BAS3231 (GenBank accession number AAT55539) encodes a protein with 71% amino acid similarity to <i>B. subtilis</i> σ<sup>1</sup> (GenBank accession number WP_032723466). The gene immediately downstream, BAS3230 (GenBank accession number AAT55538), encodes a protein with 52% amino acid similarity to the <i>B. subtilis</i> anti-sigma factor Rsgl (GenBank accession number NP_389229). We will refer to BAS3231 as <i>sigI</i> and BAS3230 as <i>rsgI</i>. A number of nearby genes are transcribed in the same orientation, raising the possibility of an operon stretching from BAS3232 to BAS3227. To determine linkage between <i>sigI</i> and surrounding genes, operon analysis was performed by RT-PCR using 34F2 RNA and sets of primers flanking the intergenic regions of each potential gene pair. As shown in Fig. 1, amplicons were obtained for the intergenic region between <i>sigI</i> and <i>rsgI</i>, indicating that transcription of these genes was linked and that they constituted an operon. BAS3232 was not linked to <i>sigI</i> and BAS3229 was not linked to <i>rsgI</i>, so <i>sigI</i> and <i>rsgI</i> constituted a two-gene operon. The downstream genes BAS3229–BAS3227 were linked, forming a three-gene operon not directly connected to <i>sigI–rsgI</i>.

To add an N-terminal His<sub>6</sub>-tag to the expressed σ<sup>1</sup>, pAW410 was transformed into <i>E. coli</i> strain BL21(DE3). Cells (2 l) were grown to OD<sub>600</sub> 0.6 and induced with 1 mM IPTG. After 2 h, cells were pelleted, resuspended in 20 ml Buffer-N (Wilson & Tan, 2002) and disrupted using an Aminco French press (Thermo Scientific). Soluble protein was separated from cell debris by centrifugation. Lysate was then loaded onto a 1 ml Ni<sup>2+</sup>-charged HiTrap Chelating HP column (GE Healthcare). The column was washed with 10 ml Buffer-N containing 10 mM imidazole followed by 10 ml Buffer-N containing 50 mM imidazole. Protein was then eluted with 5 ml Buffer-N containing 250 mM imidazole. Eluate was then loaded onto a HiPrep Sephacryl S-200 HR gel filtration column (GE Healthcare) and eluted with 360 ml Buffer-N. Fractions with the highest protein concentration were collected and concentrated with a Centiprep YM-30 column (EMD Millipore) spun for 30 min at 3000 g. Supernatant was dialysed against storage buffer (10 mM Tris/HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 100 μM EDTA, 10 mM 2-mercaptoethanol, 100 mM NaCl and 30% glycerol) overnight and again for 4 h. Protein samples were separated by SDS-PAGE and visualized by Coomassie staining. Protein concentrations were assayed by a Pierce Coomassie Protein Assay Kit (Life Technologies). Aliquots were stored at −70 °C.
An exact match to the consensus \( \sigma^1 \) promoter sequence of *B. subtilis* (Tseng et al., 2011) was identified 340 nt upstream of the translation start site of *B. anthracis* sigI. Passalacqua et al. (2009) observed two potential transcription start sites upstream of sigI in *B. anthracis*, but the transcript from the predicted \( \sigma^1 \) promoter would exceed the length cut-off value of published predictions. A potential consensus \( \sigma^A \) promoter could be identified upstream of the proximal predicted transcription start site in *B. anthracis*, consistent with observations in *B. subtilis* where sigI transcription is controlled by both the \( \sigma^1 \) and \( \sigma^A \) promoters (Huang et al., 2013; Salzberg et al., 2013; Zuber et al., 2001).

**Characterization of a sigI deletion strain**

To explore the role of sigI in *B. anthracis*, we made a markerless deletion of sigI in strain 34F2 (pXO1 \( \cdot \) pXO2 \( \cdot \)), annotated as AW-A094. Loss of sigI did not affect growth in the toxin-inducing medium LB-bic (Fig. 2a) or in non-inducing media such as LB or BHI (data not shown) when grown at 37 \( ^\circ \)C. To investigate the role of \( \sigma^1 \) in virulence gene expression, \( \beta \)-galactosidase assays using transcriptional fusions of the atxA and toxin subunit promoters to a lacZ reporter were performed. As shown in Fig. 2(b), loss of the sigI gene resulted in decreased promoter activity of pagA, which encodes the protective antigen subunit of anthrax toxin, during the stationary phase, as compared with the parental strain. Unlike the change in expression of pagA, transcription of the toxin regulatory gene atxA was unaltered. As pagA expression was reduced during the stationary phase, expression of all three toxin subunits was measured at the 8 h mark with independent replicate cultures. Promoter activity of all three toxin subunits, i.e. pagA, cya and lef, was significantly reduced, whilst atxA remained unchanged (Fig. 2c). These findings suggested that loss of sigI caused a coordinated reduction in toxin gene expression that was not the result of decreased atxA transcription.

To confirm that loss of sigI was responsible for reduced toxin gene expression, the sigI gene was restored to the chromosome of the \( \Delta \)sigI strain using plasmid pAW429, generating strain AW-A136. This strain returned sigI to the \( \Delta \)sigI strain under control of its endogenous promoters, whilst rsgI expression was retained under control of the constitutively active P_{SPAC} promoter. As shown in Fig. 2(d), pagA expression in the complemented strain was restored to parental levels whilst atxA expression remained unchanged.

**sigI is important to heat-shock survival**

In *B. subtilis*, loss of sigI results in reduced ability to withstand heat shock at 55 \( ^\circ \)C (Zuber et al., 2001); however, *B. anthracis* vegetative cells cannot grow or survive at such high temperatures. We tested the ability of 34F2 and the \( \Delta \)sigI strain to survive when grown continuously at 44 \( ^\circ \)C. As shown in Table 2, incubation of the \( \Delta \)sigI strain at 44 \( ^\circ \)C significantly reduced the number of recoverable cells relative to the parental strain, whilst the number of recoverable cells for cultures grown at 37 \( ^\circ \)C was unchanged. These data indicated that \( \sigma^1 \) contributed to the heat-shock response, consistent with observations in *B. subtilis*.

**sigI expression is induced by growth at elevated temperature**

To measure sigI promoter activity, a transcriptional fusion plasmid was constructed containing the entire 778 bp intergenic region between BAS3232 and sigI fused to promoterless lacZ. This region included both the predicted \( \sigma^1 \) and \( \sigma^A \) promoters. Consistent with the heat-shock survival results, when exposed to heat shock at 44 \( ^\circ \)C during exponential-phase growth, the growth of the parental 34F2 strain was reduced whilst growth of the \( \Delta \)sigI strain was even further reduced (Fig. 3a). At 37 \( ^\circ \)C, sigI promoter activity in the 34F2 strain was low during the exponential phase but increased as the cells entered the stationary phase (Fig. 3b) – a pattern consistent with that of virulence gene expression in *B. anthracis*. When the parental strain was shifted from 37 to 44 \( ^\circ \)C during the exponential phase, the sigI promoter was quickly induced and activity remained higher during the stationary phase. sigI promoter
Fig. 2. Growth and virulence gene expression in the sigI mutant strain. (a) Cell growth of parental and mutant strains grown in LB-bic at 37 °C under 5 % CO₂, ■, 34F2; □, ΔsigI. (b) β-Galactosidase activity in pagA and atxA reporter strains grown in LB-bic supplemented with kanamycin at 37 °C under 5 % CO₂, ■, 34F2 pagA–lacZ; •, 34F2 atxA–lacZ; □, ΔsigI pagA–lacZ; ○, ΔsigI atxA–lacZ. (c) β-Galactosidase activity of parental and mutant strains carrying reporters, as indicated, grown in LB-bic supplemented with kanamycin at 37 °C under 5 % CO₂ for 8 h post-inoculation. Filled bars, 34F2; empty bars, ΔsigI. Data were obtained from three independent cultures and represent mean ± SD. (d) β-Galactosidase activity of parental, mutant and complementation strains carrying reporters, as indicated, grown in LB-bic supplemented with kanamycin at 37 °C under 5 % CO₂ for 8 h post-inoculation. Dark grey bars, 34F2; empty bars, ΔsigI; light grey bars, sigI complementation. Data were obtained from three independent cultures and represent mean ± SD. Two-tailed unpaired t-tests: *P < 0.0001.

Table 2. Heat-shock survival

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival (c.f.u.)</th>
<th>Survival rate (%)</th>
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<tr>
<td></td>
<td>37 °C</td>
<td>44 °C</td>
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<tr>
<td>34F2 (parental)</td>
<td>2.59 × 10⁶ ± 2.21 × 10⁶</td>
<td>1.11 × 10⁶ ± 1.15 × 10⁶</td>
</tr>
<tr>
<td>AW-A094 (sigI)</td>
<td>2.56 × 10⁶ ± 3.31 × 10⁶</td>
<td>9.03 × 10⁴ ± 5.17 × 10⁴</td>
</tr>
<tr>
<td>AW-A136 (sigI)</td>
<td>2.71 × 10⁷ ± 2.71 × 10⁷</td>
<td>1.01 × 10⁶ ± 1.29 × 10⁶</td>
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activity in the ΔsigI strain was almost identical to the parental strain when grown at 37 °C. sigI promoter activity was not induced by temperature shift in the ΔsigI strain, indicating that σI was responsible for induced expression of sigI in response to heat shock. These observations were consistent with the presence of two promoters, i.e. a σI promoter induced by heat shock and a σA promoter not responsive to heat, but which was activated as the cells entered the stationary phase.

Anti-sigma factor RsgI controls sigI expression

The protein encoded immediately downstream of sigI is a predicted transmembrane protein similar to the B. subtilis anti-sigma factor RsgI. sigI and rsgI are co-transcribed as a bicistronic message. In B. subtilis, RsgI interacts with σI and regulates its activity (Asai et al., 2007). To investigate the role of the putative RsgI in B. anthracis, a markerless deletion strain, AW-A118, was generated. Growth of the ΔrsgI strain was unaltered at 37 or 44 °C relative to the parental strain (Fig. 4a). Activity of the sigI promoter was measured by β-galactosidase analysis. As shown in Fig. 4(b), loss of rsgI resulted in a strong increase in sigI promoter activity in both the exponential and stationary phases, similar to findings in B. subtilis (Asai et al., 2007) and consistent with constitutive activation of σI activity. A similar pattern of sigI promoter activity was found when strains were grown in LB-bic toxin-inducing medium (data not shown). Virulence gene expression was also assayed in the ΔrsgI strain by β-galactosidase analysis under toxin-inducing conditions, and no difference was seen in growth or expression of pagA, lef, cya and atxA (Fig. 4c). These data indicated that RsgI controlled activity and expression of σI, but did not influence virulence gene expression.

Characterization of sigI–rsgI double-deletion strain

Loss of sigI, but not rsgI, resulted in decreased virulence gene expression. To confirm the predicted roles of σI and RsgI in B. anthracis, a double-gene-deletion strain, AW-A125, missing both the sigma factor and its cognate anti-sigma factor, was constructed. The double gene deletion of sigI–rsgI displayed no growth defect when grown at 37 °C under toxin-inducing conditions (Fig. 5). Virulence promoter activity was reduced for pagA, cya and lef, but atxA promoter activity was unchanged, similar to the phenotypes of a deletion of sigI alone. These observations were consistent with our previous findings and suggested no role for RsgI in virulence regulation in the absence of σI.

σI-containing RNAP does not initiate transcription from the pagA or atxA promoter regions

The decrease in toxin gene expression associated with loss of sigI could be explained if σI directs transcription of
Toxin genes. To test this hypothesis, in vitro transcription was performed using $\sigma^1$-containing RNAP on the pagA, atxA and sigI promoter regions. Whilst a predicted $\sigma^1$ promoter could not be identified upstream of either atxA or pagA, a strong match to the B. subtilis consensus $\sigma^1$ promoter was found upstream of sigI. DNA template for sigI from −2757 to +114 relative to the translation start site contains the entire intergenic region between BAS3232 and sigI, including the predicted $\sigma^1$ promoter and the mapped 5′ transcript ends near a predicted $\sigma^A$ promoter (Passalacqua et al., 2009). DNA templates for atxA from −945 to +169 and pagA from −151 to +189 relative to the translation start site contain their previously identified promoters (Bongiorni et al., 2008; Dai et al., 1995; Koehler et al., 1994). Control in vitro transcription reactions using either purified B. anthracis $\sigma^1$ or E. coli RNAP core alone did not result in a detectible transcript. The reaction containing $\sigma^1$, RNAP core and the sigI promoter DNA template generated ~450 bases, consistent with transcripational initiation from the predicted $\sigma^1$ promoter (Fig. 6). Reactions containing $\sigma^1$-RNAP holoenzyme using the pagA or atxA templates did not result in detectable transcripts. B. anthracis $\sigma^1$ appeared to direct transcription of its own promoter, but could not direct transcription of either atxA or pagA.

**DISCUSSION**

B. anthracis $\sigma^1$ influences toxin gene expression under non-heat-shock, toxin-inducing conditions that mimic growth within the mammalian host, but the mechanism of regulation remains unknown. That the promoter activity of all three toxin subunits (pagA, cya and lef) was coordinately downregulated in the ΔsigI strain suggests a role of the master virulence regulator AtxA in this process. Transcription of atxA was unaffected in the ΔsigI strain and $\sigma^1$-RNAP does not appear to directly transcribe atxA,
suggesting a post-transcriptional mechanism of AtxA regulation. Several factors have been shown to influence atxA transcription (Chiang et al., 2011; Dale et al., 2012; Saile & Koehler, 2002; Wilson et al., 2009), but relatively little is known about post-transcriptional regulation. The phosphorylation and dimerization status of AtxA strongly influences its activity (Hammerstrom et al., 2011, 2015; Tsvetanova et al., 2007) and the global regulator CodY regulates AtxA post-translationally. Whether loss of σ^I affects AtxA activity through these mechanisms or through an unknown mode of post-transcriptional regulation is currently unknown.

σ^I regulates a number of processes in B. subtilis, the best studied of which is the control of cell wall synthesis and remodelling during cell growth and division (Huang et al., 2013; Salzberg et al., 2013; Tseng & Shaw, 2008; Tseng et al., 2011). σ^I-RNAP directly controls the expression of several genes associated with cell wall metabolism, such as LytE, MreBH and BcrC, through initiation of transcription from
sigI and rsgI are expressed as a bicistronic message. Loss of RsgI resulted in increased sigI promoter activity at both 37 and 44°C, indicating that RsgI controls σ1 activity under non-heat-shock conditions, similar to B. subtilis (Asai et al., 2007). Loss of rsgI did not alter growth under heat-shock conditions, suggesting that neither the loss of RsgI nor the strong increase in sigI expression significantly alters growth under heat stress. In B. subtilis, the N-terminal domain of the RsgI interacts directly with σ1 protein. Upon exposure to heat, σ1 is released from RsgI and activated by heat through DnaK-mediated and independent processes before integration into the σ1–RNAP holoenzyme (Asai et al., 2007). A similar process may be at work in B. anthracis, and further investigation is required to understand the role of RsgI and DnaK in σ1 regulation. When grown under toxin-inducing conditions, sigI–rsgI promoter activity was still strongly increased in the ΔrsgI strain relative to the parental strain (data not shown), but virulence gene promoter activity was unaltered. Toxin gene expression in the ΔsigI–rsgI strain was reduced similarly to the ΔsigI strain. These observations suggest that RsgI contributes to activation of σ1 under stress conditions, but the role of RsgI in responding to signals associated with induction of virulence gene expression to alter σ1 activity is unclear.

Highly elevated temperatures are unlikely to be directly relevant to toxin production during the course of an infection in the mammalian host, as toxin gene expression occurs primarily under non-heat-shock conditions. The role of σ1 in the heat-shock response may be relevant to the pathogenic life cycle of B. anthracis if vegetative cells are exposed to increased temperatures prior to completion of sporulation. Loss of σ1 does not affect sporulation efficiency (data not shown). Some evidence suggests vegetative growth of B. anthracis in the external environment (Salie & Koehler, 2006) where changes in gene expression in response to temperature might also be relevant. Interestingly, loss of another temperature-responsive alternative sigma factor, σB, does alter virulence of B. anthracis in an animal model of infection, although the mechanism is not understood (Fouet et al., 2000). The role of heat-shock response pathways in virulence and spread of B. anthracis may require further investigation.

sigI–rsgI transcription seems to occur from both the σ1 and σA promoters. In vitro transcription with σ1-containing RNAP generated a transcript sized consistently with transcriptional initiation from the predicted σ1 promoter. Passalacqua et al. (2009) observed potential transcription start sites upstream of sigI under toxin-inducing conditions that are much closer to the translation start codon than the σ1 promoter (Passalacqua et al., 2009). A potential σA promoter was identified upstream of the proximal transcription start site, consistent with observations in B. subtilis where sigI transcription is controlled by both the σ1 and σA promoters (Huang et al., 2013; Salzberg et al., 2013; Zuber et al., 2001). In B. subtilis, the σ1 promoter is repressed and the σA promoter is activated in unstressed, exponentially growing cells (Salzberg et al., 2013). The pattern of sigI–rsgI induction in B. anthracis under toxin-inducing conditions is similar to that of σA-controlled pagA expression (Koehler et al., 1994). These observations are consistent with a model of sigI–rsgI regulation under virulence conditions primarily through a σ1-controlled promoter, and sigI–rsgI regulation under stress conditions primarily through a σA-controlled promoter.

This report demonstrates a connection between σ1 activity and the regulation of virulence gene expression in B. anthracis. Further work is required to identify the genes directly regulated by σ1 and how altering the regulation of these targets affects virulence gene expression. It is also uncertain whether the reduction in toxin promoter activity described or any other effects associated with loss of σ1 would alter the pathogenesis of B. anthracis in animal models of infection.

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