Alternate response of *Bacillus anthracis atxA* and *acpA* to serum, HCO_3^- and CO_2

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Abstract

Bacillus anthracis overcomes host immune responses by producing capsule and secreting toxins. Production of these virulence factors in response to entering the host environment was shown to be regulated by *atxA*, the major virulence regulator, known to be activated by HCO_3^- and CO_2. While toxin production is regulated directly by *atxA*, capsule production is independently mediated by two regulators; *acpA* and *acpB*. In addition, it was demonstrated that *acpA* has at least two promotors, one of them shared with *atxA*. We used a genetic approach to study capsule and toxin production under different conditions. Unlike previous works utilizing NBY-HCO_3^- medium under CO_2 enriched conditions, we used a sDMEM-based medium. Thus, toxin and capsule production can be induced in ambient or CO_2 enriched atmosphere. Using this system, we could differentiate between induction by 10% NRS, 10% CO_2 or 0.75% HCO_3^-. In response to high CO_2, capsule production is induced by *acpA* in an *atxA*-independent manner, with little to no toxin (protective antigen PA) production. *atxA* is activated in response to serum independently of CO_2 , inducing toxin and capsule production in an *acpA* or *acpB* dependent manner. HCO_3^- was also found to activate *atxA* , but in non-physiological concentrations. Our findings may help explain the first stages of inhalational infection, in which spores germinating in dendritic cells require protection (by encapsulation) without affecting cell migration to the draining lymph-node by toxin secretion.

Introduction

Bacillus anthracis, the causative agent of anthrax, is a gram positive, spore forming bacterium that infects humans via three major routes; inhalational (lung), cutaneous (skin) and gastrointestinal (gut) [1, 2]. The infectious form of *B. anthracis* is the spore, durable and environmentally stable for decades. In response to entering the host, spores germinate and overcome the immune system utilizing two major virulence factors; the poly-δ-D-glutamic antiphagocytic capsule and the tripartite toxin system [3]. Capsule producing genes are encoded as a polycistronic gene cluster on the pXO2 virulence plasmid, comprised of the *capB,C,A,D,E* genes. CapB and CapC are the D-glutamic acid polymerization enzymes, while CapA and CapE form the transport channel that exports the polymer from the cytoplasm through the cell wall and membrane to the cell surface [4-6]. Once there, CapD hydrolyzes the polymer to shorter chains and covalently links them to the cell wall [7]. CapD also regulates the length of the bound polymer chains through its hydrolysis activity [4, 8, 9]. The tripartite toxins are encoded by the *pag* (protective antigen-PA), *lef* (lethal factor-LF) and *cya* (edema factor-EF) genes, located on the pXO1 virulence plasmid [1]. LF is a metalloprotease that specifically cleaves members of the MAP kinase regulatory pathway of mammalian cells [10]. EF is a potent calmudolin dependent adenylate cyclase that interferes with cell regulation by elevating the internal cAMP levels [11]. Both LF and EF are driven into mammalian cells by PA, which binds specific receptors ANTXR1 (TEM8) and ANTXR2 (CMG2) [12]. Following binding to the receptor, PA is processed by cell-associated furin, activating oligomerization, forming a heptamer that binds LF and EF [12, 13]. The complex is then phagocyted and upon lysosomal fusion, the pH drop causes a conformational shift resulting in LF and EF injection into the cytoplasm. These toxin moieties then disrupt cell activity by disrupting intra-cellular signaling. Intoxication of immune cells results in their inactivation, disrupting host immune responses [12].

The AtxA was shown to be the major virulence regular of *B. anthracis* [2, 14]. This pXO1-located gene (encoding for the AtxA protein) activates a cascade of regulatory processes, resulting in up and down regulation of both chromosomal and plasmid encoded genes [15]. Capsule production is regulated by two pXO2 encoded genes – *acpA* [15] and *acpB* [16, 17]. Though it was demonstrated that AtxA regulates these two genes [18], it is not essential for capsule production since ΔpXO1 strains remain capable of capsule production [19, 20]. Since *B. anthracis* does not produce toxins or capsule under normal laboratory growth conditions, specific host-simulating growth conditions were developed. It was reported that growth of *B. anthracis* in NBY broth supplemented with glucose and bicarbonate in 5-15% CO_2 atmosphere induces toxin secretion and capsule production [16, 20-22]. These growth conditions were used to study different aspects of virulence regulation in *B. anthracis*.

75 Resulting findings indicated that AtxA in induced in response to HCO_3^- and CO_2, activating both toxin
76 and capsule production. This concept implies that toxins and capsule are produced simultaneously in the
77 host.
78 Previously we reported that growth of *B. anthracis* in supplemented DMEM (a high glucose cell culture
79 medium supplemented with pyruvate, glutamine, nonessential amino acids, (henceforth sDMEM) with the
80 addition of 10% serum, and incubated in a 10% CO_2 atmosphere, induces virulence factor production [17,
81 19]. These conditions were used to examine the effects of serum (here - normal rabbit serum, NRS),
82 HCO_3^- and/or CO_2 atmosphere, on the regulation of toxin and capsule production. This was coupled with a
83 systematic genetic approach, to dissect the regulation of the bacterial virulence response. Unlike previous
84 reports, we show that *AtxA* is induced by NRS or HCO_3^-, but not CO_2. The capsule regulator AcpA can be
85 induced by CO_2 in an *atxA*-independent manner, or by NRS in an *atxA*-dependent manner. AcpB was
86 activated only in an *atxA*-dependent manner, with *atxA* deletion resulting in complete abrogation of *acpB*
87 capsule regulation. Our results indicate that bacteria is capable of independent capsule production,
88 uncoupled from toxin production, but that toxins are always be co-induced with the capsule.
89

Materials and Methods

Bacterial strains, media and growth conditions.

Bacterial strains used in this study are listed in **Table 1**. For the induction of toxins and capsule production, we employed a modified DMEM (supplemented with 4mM L-glutamine, 1 mM Sodium pyruvate, 1% non-essential amino acid) that was supplemented with 10% normal rabbit serum or 0.75% NaHCO3 (Biological Industries – Israel) – hence sDMEM. Spores of the different mutants were seeded at a concentration of $5x10^5$ CFU/ml and grown in 96 well tissue culture plates (100µl per well) for 5 or 24h at 37oC in ambient or 10% CO2 atmosphere.

	Description/characteristics	Source
Strain		
B. anthracis		
Vollum	ATCC 14578	IIBR collection
VollumΔpXO1	Complete deletion of the plasmid pXO1	IIBR collection
VollumΔ*atxA*	Complete deletion of the *atxA* gene	This study
VollumΔ*acpA*	Complete deletion of the *acpA* gene	[17]
VollumΔ*acpB*	Complete deletion of the *acpB* gene	[17]
VollumΔ*acpA*Δ*acpB*	Complete deletion of the *acpA* and *acpB* genes	[17]
VollumΔ*pag*Δ*cya*Δ*lef* (ΔTox)	Complete deletion of the *pag*, *lef* and *cya* genes	[23]
VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpA*	Complete deletion of the *acpA* gene in the VollumΔ*pag*Δ*cya*Δ*lef* mutant	[17]
VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpB*	Complete deletion of the *acpB* gene in the VollumΔ*pag*Δ*cya*Δ*lef* mutant	[17]
VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpA*Δ*acpB*	Complete deletion of the *acpA* and *acpB* genes in the VollumΔ*pag*Δ*cya*Δ*lef* mutant	[17]
VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpA*Δ*atxA*	Complete deletion of the *atxA* gene in the VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpA* mutant	This study
VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpB*Δ*atxA*	Complete deletion of the *atxA* gene in the VollumΔ*pag*Δ*cya*Δ*lef*Δ*acpB* mutant	This study
VollumΔpXO2 Δ*bclA*:: pag_{prom}::*capA-E*	Genomic insertion of the *pagA* promotor in front of the CAP operon replacing the *bclA* gene in the VollumΔpXO2	[17]
VollumΔpXO2 Δ*bclA*:: pag_{prom}::*capA-E*Δ*atxA*	Deletion of the *atxA* gene from the strain having Genomic insertion of the *pagA* promotor in front of the CAP operon replacing the *bclA* gene in the VollumΔpXO2	[17]

Table 1: Bacterial strains used in this study

102 **Mutant strain construction.**

103 Oligonucleotide primers used in this study were previously described [17, 19, 23]. The oligonucleotide
104 primers were designed according to the genomic sequence of *B. anthracis* Ames strain. Genomic DNA
105 (containing the chromosomal DNA and the native plasmids, pX01 and pX02) for cloning the target gene
106 fragments was extracted from the Vollum strain as previously described [24]. Target genes were
107 disrupted by homologous recombination, using a previously described method [25]. In general, gene
108 deletion or insertion was accomplished by a marker-less allelic exchange technique that replaced the
109 complete coding region with the SpeI restriction site or the desired sequence. At the end of the
110 procedure the resulting mutants did not code for any foreign sequences and the only modification is the
111 desired gene insertion or deletion. Deletion of the atxA gene was performed as previously described [25].
112 All the mutants were tested for their ability to produce capsule by incubation in sDMEM. The capsule was
113 visualized by negative staining with India ink.

114 **Toxin quantification**

115 Protective antigen (PA) concentration was determine by capture ELISA using the combination of a
116 polyclonal and monoclonal αPA antibodies previously described [23]

117

118 ## Results

119 **The effect of supplements and growth condition on capsule production in sDMEM medium.** The basic
120 medium used to test capsule and toxin induction was high glucose DMEM supplanted with glutamine,
121 pyruvate and non-essential amino acids, hence sDMEM. We first tested the effect of growth media
122 volume on capsule production in a 96-well tissue culture microplate format. This was done to allow higher-
123 throughput testing of growth conditions and genetic manipulations. We inoculated *B. anthracis* Vollum
124 spores ($5x10^5$ CFU/ml) into 100μl, 200μl and 300μl sDMEM at 37°C in ambient atmosphere for 24h. No
125 capsule production could be detected in 100μl sDMEM (**Figure 1**). However, increasing the culture volume
126 resulted in partial (200μl) or full (300μl) capsule production (**Figure 1**). These findings could be possibly
127 explained by higher CO_2 concentrations reached in the well when bacteria are grown in larger volumes,
128 due to a lower surface area to volume ratio, limiting gas exchange. This is coupled with a larger volume of
129 multiplying and respiring bacteria, further increasing CO_2 concentrations. This hypothesis is supported
130 by the finding that growth in 10% CO_2 atmosphere does induce capsule production in 100μl sDMEM (**Figure**
131 **1**). Having established non-capsule inducing growth conditions for our experiments, we next tested the
132 effect of adding normal rabbit serum (NRS) to growth media. NRS was shown to induce capsule
133 production, regardless of CO_2 concentrations in the incubator's atmosphere, with all the bacteria grown
134 with NRS encapsulated in ambient or 10% CO_2 atmosphere (**Figure 1**).
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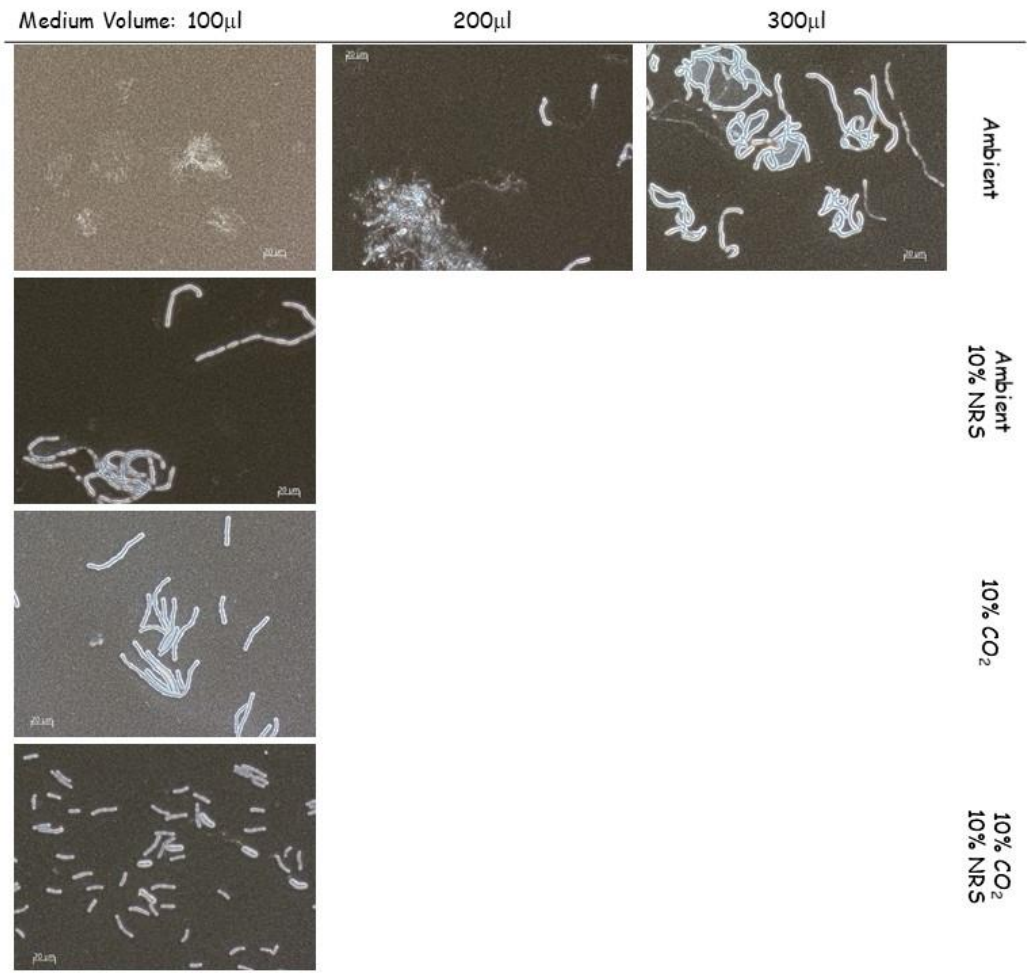

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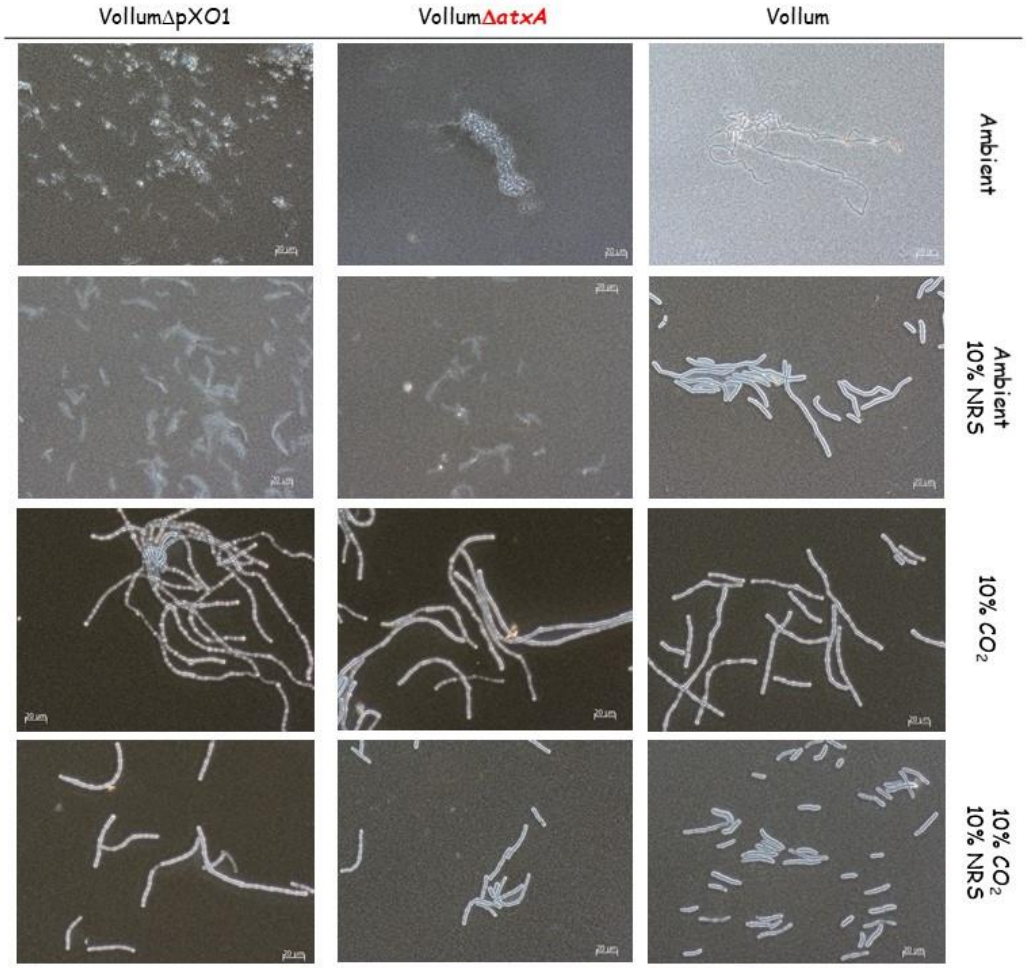
137

138 **Figure 1. The effect of growth conditions on capsule induction by *B. anthracis* Vollum.** Spores were
139 seeded into 100µl, 200µl or 300µl of sDMEM and incubated at 37°C in an ambient atmosphere (upper panel) for 24h.
140 To examine the effect of serum or CO_2 on capsule production, spores were seeded into 100µl sDMEM or sDMED-
141 NRS (supplemented with 10% NRS). Samples were incubated at 37°C in under ambient or 10% CO_2 atmosphere (see
142 right hand side legend) for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical
143 bright outer layer).

144

145 **The role of *atxA* on capsule production and toxin secretion under different growth conditions**. To
146 determine the role of *atxA* on capsule and toxin production, we compared capsule production of the wild
147 type Vollum strain with the ΔpXO1 or Δ*atxA* mutants (**Table 1**) following growth in sDMEM under the
148 different growth conditions (NRS/CO_2 presence). None of the strains produced capsule under ambient
149 atmosphere in un-supplemented sDMEM (**Figure 2**). Addition of 10% NRS, under ambient atmosphere,
150 induced capsule production by the wild type Vollum strain but not by the *atxA* null mutants (both ΔpXO1
151 or Δ*atxA*, **Figure 2**). Under 10% CO_2, all three strains produced capsule. We therefore conclude that the
152 response to NRS is *atxA* dependent, as lacking either the atxA gene or the entire pXO1 plasmid abrogates

153 this response (**Figure 2**). These results also demonstrate the presence of another, atxA independent
154 mechanism, responding to CO_2. However, the effect of NRS in the presence of CO_2 on capsule production
155 under these conditions could not be resolved.



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157 Figure 2. Capsule production of the Vollum wild type, ΔpXO1 or Δ*atXA* mutants under different growth
158 conditions. Spores of the wild type and different mutants (top panel) were seeded into 100μl of sDMEM. The same
159 strains were grown in sDMEM supplemented with 10% NRS (second row). The same conditions were applied in the
160 presence of 10% CO_2 (lower two rows) for 24h. Capsule was imaged by India ink negative staining (capsule presence
161 forms a typical bright outer layer).

162

163 As *atxA*, the major virulence regulator, regulates toxin expression (LF, EF and PA), we tested their
164 secretion into growth medium after growth, both for the Vollum and the VollumΔ*atxA* mutant (the ΔpXO1
165 mutant lacks the entire set of toxin genes and is therefore irrelevant for such a test). Toxins secretion
166 was determined by ELISA for the most abundant component, PA. For the VollumΔ*atxA* mutant, PA was
167 undetectable, both under 10% CO_2, with 10% NRS and in the presence of both. These results confirm the
168 role of atxA in toxin expression regulation (**Table 2**). For the wild type Vollum, no toxins were induced in
169 sDMEM alone coupled with under ambient atmosphere. The addition of 10% NRS alone induced high PA

170 expression. sDMEM without NRS under 10% CO_2 again did not induce PA expression (**Table 2**). However,
171 the addition of both NRS and CO2 resulted in reduced PA expression (about a 70% reduction) (**Table 2**).
172 This finding was maintained throughout different, independent experiments. However, we cannot exclude
173 the possibility that this difference resulted from growth rates variation under the tested growth
174 conditions. No PA secretion could be detected in the *atxA* null mutant under any of the tested growth
175 conditions. Our finding that 10% CO_2 atmosphere in itself does not activate *atxA*-dependent toxin
176 expression is surprising, since it was repeatedly demonstrated such activation is achievable by HCO_3^-
177 addition. We therefore tested both capsule production and toxin expression in sDMEM supplemented
178 with 0.75% HCO_3^- under 10% CO_2 atmosphere. These atmospheric conditions were chosen since HCO_3^- in
179 aqueous media is in equilibrium with CO_2 and H_2O, dramatically increasing the levels of the soluble CO_2 in
180 the medium even under ambient atmosphere, and we sought to expose our experimental controls to
181 conditions as similar as possible.
182
183 Table 2. Protective Antigen secretion of Vollum or Vollum∆*atxA* under different growth conditions.

	Vollum∆*atxA*	Vollum	Atmosphere	Supplements
PA µg/ml	<0.1	<0.1	Ambient	-
	<0.1	8.7	Ambient	10% NRS
	<0.1	<0.1	10% CO_2	-
	<0.1	2.6	10% CO_2	10% NRS

184
185
186 According to our previous findings, capsule production is CO_2 dependent. Therefore, we found that the
187 above tested strains were encapsulated under the conditions applied (**Figure 3**). PA secretion was tested
188 only for Vollum and Vollum∆*atxA*. Supplementing the sDMEM with 0.75% HCO_3^- induced PA secretion by
189 the Vollum strain but not by the Vollum∆*atxA* mutant. This indicates that HCO_3^- induces toxin secretion
190 in an *atxA* dependent manner (**Table 3**). To validate these findings, we used a previously reported
191 Vollum∆pXO2 chimera in which we substituted the genomic *bclA* gene (which is a redundant exosporium
192 glycoprotein) with a CAP operon altered to be regulated by the PA_{prom} (**Table 1**). Thus, this mutant serves
193 here as an indicator for AtxA dependent activation of the PA promotor, which results in capsule
194 production (assayable readout). This mutant was then used as the basis for another mutant strain, in
195 which the *atxA* gene was deleted, on top of the existing genetic alterations (**Table 1**). Thus, the exact
196 role *atxA* plays in the regulation on the PA-promotor under the tested conditions could be resolved.
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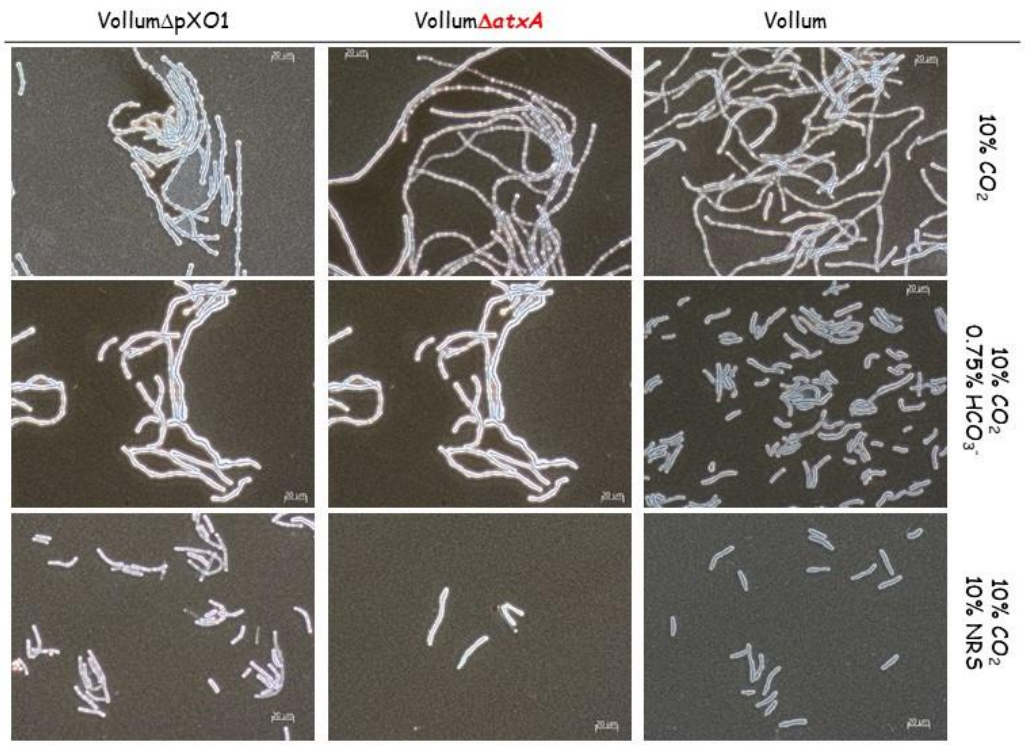


Figure 3. Effect of 0.75% HCO_3^- on capsule production of Vollum, VollumΔ*atxA* or VollumΔpXO1. Spores were seeded into 100µl of sDMEM as is or supplemented with 0.75% HCO_3^- or 10%NRS, and incubated at 37°C under 10% CO_2 for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical bright outer layer).The different mutations are indicated in the top panel.

Table 3. PA secretion by Vollum and VollumΔ*atxA* in sDMEM supplemented with HCO_3^-.

	VollumΔ*atxA*	Vollum	Atmosphere	Supplements
PA µg/ml	<0.1	<0.1	10% CO_2	-
	<0.1	3.12	10% CO_2	0.75% HCO_3-
	<0.1	5.3	10% CO_2	10% NRS

While 10% CO_2 atmosphere induced capsule production in the Vollum wild type strain, little or no capsule production could be detected in the VollumΔpXO2Δ*bclA*::*pagA*$_{prom}$-capA-E chimeric strain (**Figure 4**). Supplementing sDMEM with 0.75% HCO_3^- resulted in capsule production in the Vollum and VollumΔpXO2Δ*bclA*::*pagA*$_{prom}$-capA-E chimera. However, deletion of the *atxA* gene, in the background of the chimera mutant described, resulted in loss of capsule production (VollumΔpXO2Δ*bclA*::*pagA*$_{prom}$-capA-EΔ*atxA*, **Figure 4**). Similar results were obtained by supplementing sDMEM with 10% NRS. These results

complement the PA secretion experiment, indicating that HCO_3^- and NRS induce toxin secretion in an *atxA* dependent manner.

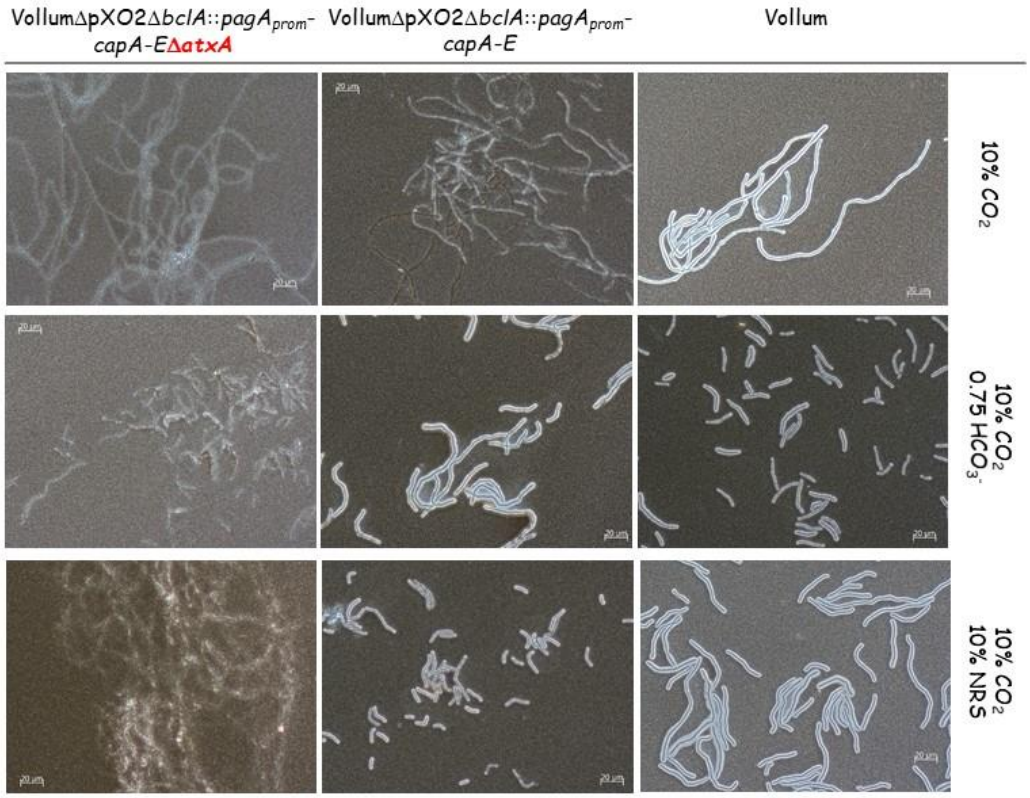
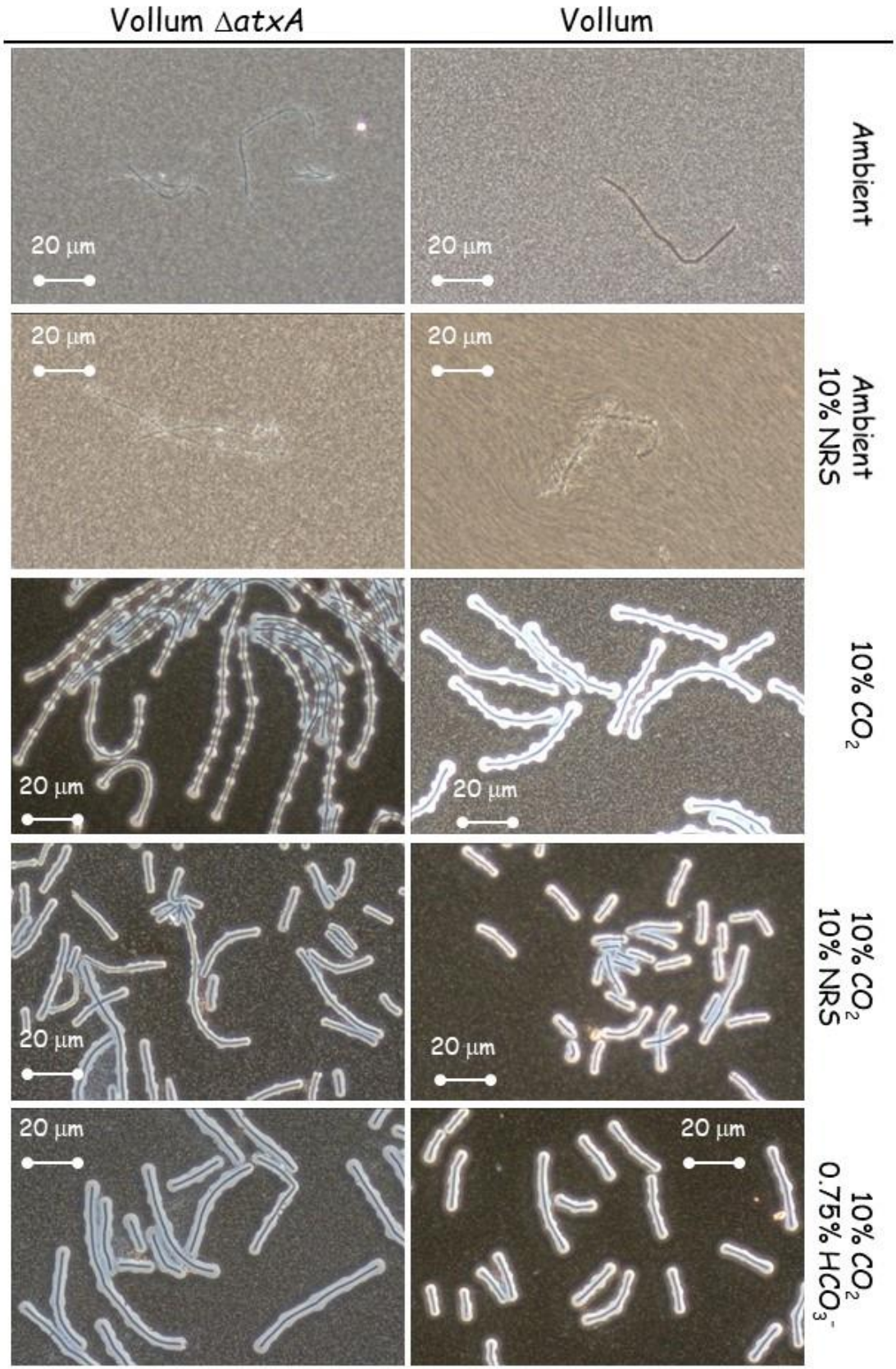


Figure 4. Capsule production in response to HCO_3^- in a PA_{prom}- regulated genomic CAP operon. Spores were seeded into 100µl of sDMEM as is or supplemented with 0.75% HCO_3^- or 10% NRS and incubated at 37°C under an atmosphere of 10% CO_2 for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical bright outer layer).The different mutations are indicated (top panel).

The effect of short incubations on capsule and toxins production in response the different growth conditions. Aerobic growth affects different parameters of the liquid medium such as pH and O_2/CO_2 concentrations, especially when the bacteria reaches high concentration (CFU/ml). As was shown previously [26], capsule production and toxin secretion can be detected as early as 2-5h of growth in sDMEM under 10% CO_2 atmosphere. To minimize changes in media conditions resulting from bacterial growth, we examined capsule production and PA secretion after 5h growth in different growth conditions. Vollum growth under ambient atmosphere did not result in any capsule accumulation or toxin secretion following 24h incubation (**Figure 2, Table 2**). This was true also for a short (5h) incubation (**Figure 5, Table 4**). Supplementing the media with 10% NRS induced capsule production and PA secretion following 24h incubation under ambient atmosphere (**Figure 2, Table 2**). A shorter (5h) incubation induced measurable PA secretion (**Table 4**) but no capsule production (**Figure 5**). This PA secretion is AtxA dependent, as deletion of the *atxA* gene resulted in no PA accumulation following 5h (**Table 4**) or 24h

233 incubation (**Table 2**). Incubating the bacteria in 10% CO_2 atmosphere for 5h resulted in capsule production
234 (**Figure 5**), similarly to that observed following a 24h incubation (**Figures 1-3**). This capsule accumulation
235 was AtxA independent and was not significantly affected by the addition of 10% NRS or HCO_3^- (**Figure**
236 **5**). PA secretion was not induced by 10% CO_2 in itself, but required addition of NRS or HCO_3^- (**Table 4**),
237 however unlike the 24h incubation (**Table 3**), the amount of PA secreted in response to HCO_3^- was
238 significantly less (~10%) than of that induced by NRS (**Table 4**). Under all conditions the PA secretion
239 was AtxA dependent.



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241 Figure 5. Effect of deleting *atxA* on capsule production after a short (5h) incubation under different

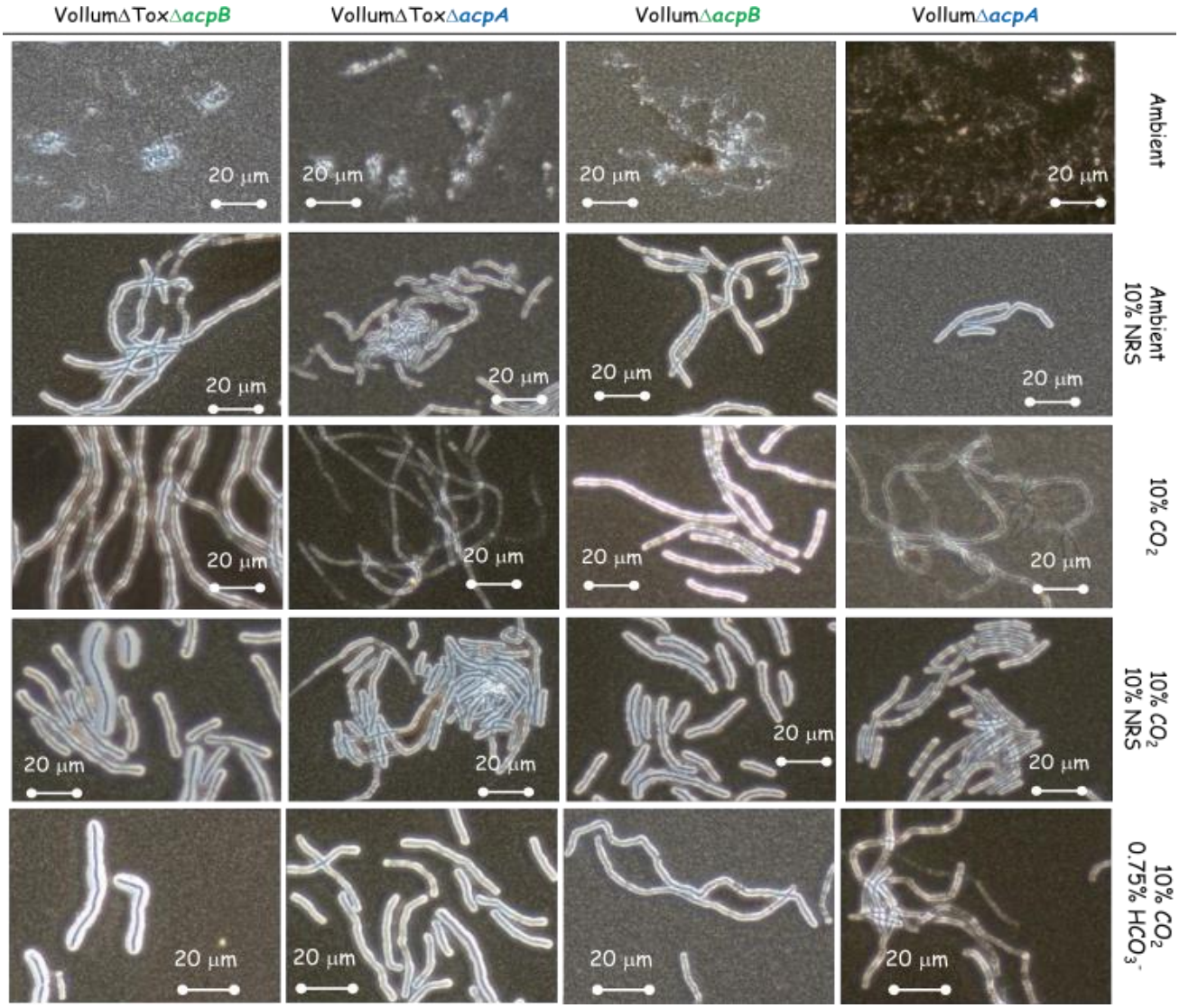
242 growth conditions. Spores of the wild type and Δ*atxA* mutant (top panel) were seeded into 100µl of sDMEM as is

243 or supplemented with 10% NRS and incubated at 37°C in an ambient or 10% CO_2 atmosphere (as indicated on the
244 right) for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical bright outer layer).
245
246 Table 4. PA secretion by Vollum and Vollum*ΔatxA* following 5h growth in different conditions

	Vollum*ΔatxA*	Vollum	Atmosphere	Supplements
PA µg/ml	<0.1	<0.1	Ambient	-
	<0.1	1.89	Ambient	10% NRS
	<0.1	<0.1	10% CO_2	-
	-	4.25	10% CO_2	10% NRS
	-	0.29	10% CO_2	0.75% HCO_3-

247
248 **Regulation of *acpA* and *acpB* in response to different growth conditions.** Toxin production is induced
249 in an *atxA* dependent manner in response to HCO_3^- or NRS, while capsule production is also induced by a
250 CO_2 enriched (10%) atmosphere. Capsule production is known to be regulated by two regulatory proteins,
251 AcpA and AcpB. We therefore tested the effect of deleting each of these genes on capsule production
252 in response to different growth conditions. The complete coding region of *acpA* or *acpB* was deleted
253 independently in the background of wild type Vollum or the toxin deficient mutant - VollumΔTox
254 (VollumΔ*pag*Δ*cya*Δ*lef* **Table 1**). As was previously shown for the wild type Vollum strain (**Figure 2**), none
255 of these mutants produced capsule following growth in sDMEM under ambient atmosphere (**Figure 6**). The
256 presence of either *acpA* or *acpB* is sufficient for capsule production in sDMEM supplemented with 10%
257 NRS, regardless to the presence or absence of 10% CO_2 atmosphere (**Figure 6**). However, in the absence
258 of NRS, only AcpA expressing mutants (lacking *acpB*) produce significant capsule when grown in 10% CO_2
259 atmosphere. Adding 0.75% HCO_3^- to sDMEM induced capsule production in the presence of either *acpA*
260 or *acpB*. Mutants lacking *acpA* (expressing only *acpB*), did not produce significant capsule in 10% CO_2
261 atmosphere. To examine the role of *atxA* in these processes, we deleted the *atxA* gene in the background
262 of our VollumΔToxΔ*acpA* or Δ*acpB* mutants. Deleting *atxA* in the VollumΔToxΔ*acpA*, expressing *acpB*,
263 abolished capsule production under all tested conditions (**Figure 7**). However, deleting *atxA* in the
264 VollumΔToxΔ*acpB*, expressing *acpA*, did not affect capsule accumulation, compared to the AtxA
265 expressing mutant. This finding indicates that *acpA* operates in an AtxA independent manner (**Figure 7**).
266 As we demonstrated (**Figure 2**), capsule production could be induced in ambient atmosphere by adding
267 10% NRS to sDMEM. This induction is AtxA dependent, since no capsule production was detected in the
268 VollumΔ*atxA* mutant under these conditions (**Figure 2**). Since AcpA dependent capsule production in 10%
269 CO_2 atmosphere was AtxA independent, we tested the role of AtxA on AcpA dependent capsule
270 production in response to 10% NRS in ambient atmosphere. As 10% NRS induced capsule production of
271 VollumΔ*acpB* under ambient atmosphere (**Figure 6, Figure 8**) we tested the effect of *atxA* deletion on

272 capsule production under these conditions. Unlike the CO_2 induction, under ambient atmosphere, AcpA
273 dependent capsule production in response to NRS is AtxA dependent (**Figure 8**).



274

275 Figure 6. The effect of absence of *acpA* or *acpB* on capsule production in response to 10% NRS under
276 ambient or 10% CO_2 atmosphere. Spores of the Δ*acpA* or *acpB* mutants (top panel) were seeded into 100µl of
277 sDMEM as is or supplemented with0.75% HCO_3^- or 10% NRS and incubated at 37°C in an ambient or 10% CO_2
278 atmosphere (as indicated on the right) for 24h. Capsule was imaged by India ink negative staining (capsule presence
279 forms a typical bright outer layer).

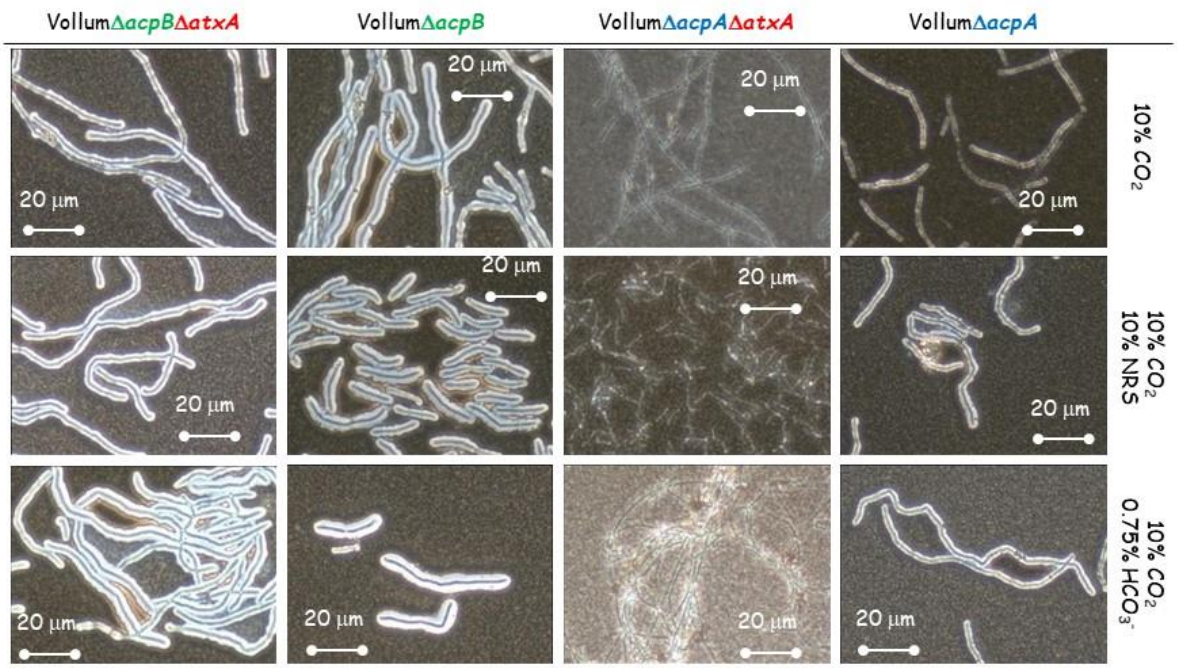


Figure 7. Effect of AtxA on capsule production in the presence of either AcpA or AcpB. Spores of the different mutants (top panel) were seeded into 100µl of sDMEM as is or supplemented with 0.75% HCO_3^- or 10% NRS and incubated at 37°C in 10% CO_2 atmosphere (as indicated on the right) for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical bright outer layer).

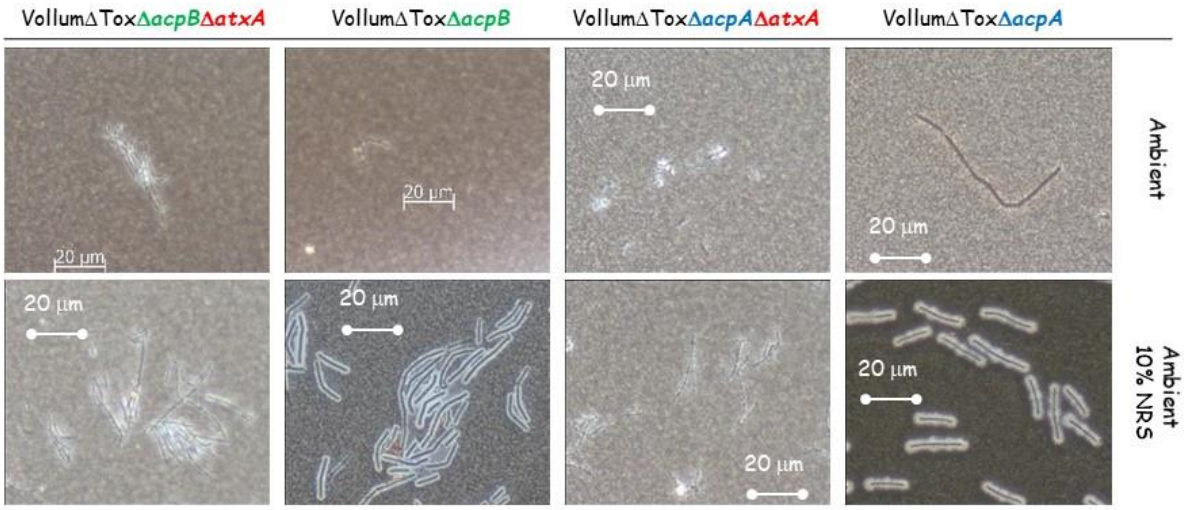


Figure 8. Effect of AtxA on capsule production in response to 10% NRS in ambient atmosphere. Spores of the different mutants (top panel) were seeded into 100µl of sDMEM as is or supplemented with 10% NRS and incubated at 37°C in ambient atmosphere (as indicated on the right) for 24h. Capsule was imaged by India ink negative staining (capsule presence forms a typical bright outer layer).

292 **Gas content of the supplemented and basic DMEM media under the different growth conditions.** Gas
293 content was determined by Abbott iSTAT blood gas analyzer. The different media were incubated at
294 37°C in ambient or 10% CO_2 atmosphere for 4 h and analyzed using the EC8+ cartridge. The results shown
295 in **Table 5** indicate that the main parameter that is affected by the 10% CO_2 is the soluble CO_2 (PCO_2)
296 rather than HCO_3^-. Unlike the HCO_3^- levels that are higher under all growth conditions from those
297 considered normal for human blood, the PCO_2 levels in the presence of 10%CO_2 are well within or very
298 close to normal values.
299
300 **Table 5. Gas analysis of the sDMEM media under the different growth conditions**

10% CO_2	-	+	+	-	+	Normal arterial	Normal venous
Supplement	-	-	10% FCS	10% FCS	0.75% HCO_3 1:1*		
pH	8.0	7.691	7.447	8.149	7.972	7.35-7.45	7.31-7.41
PCO_2 (mmHg)	18.4	34.9	58.1	14.2	34.3	35-45	41-51
PO_2 (mmHg)	230	148	126	173	183	80-100	35-40
HCO_3 (mmol/L)	48.7	42.2	40.1	49.5	79.2	22-26	22-26
sO_2 (%)	100	100	99	100	100	95-100	/

301 *at the end of the 4h incubation the media was diluted 1:1 in sDMEM prior to the analysis since the
302 undiluted media was off scale.
303

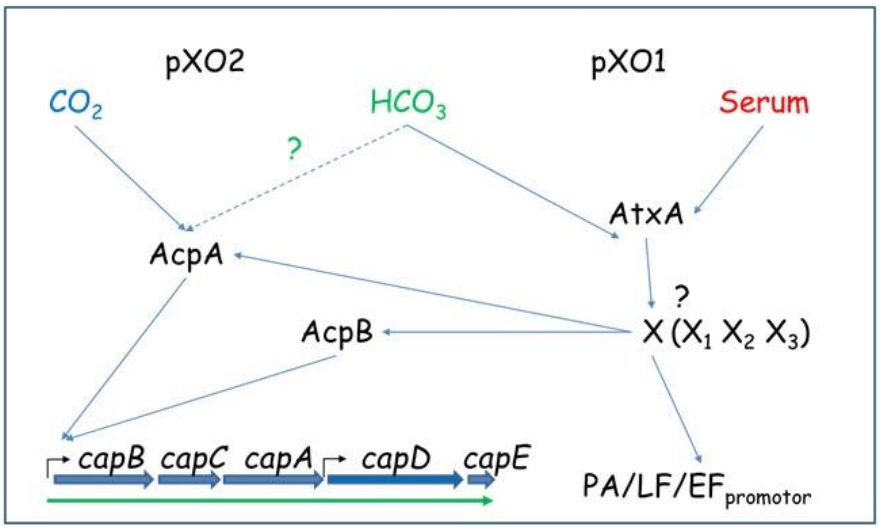
304 ## Discussion

305 Successful invasion requires the pathogen regulate its virulence factors in a way that will maximize their
306 effect on host defense mechanisms. The trigger for such activation is usually host derived. This can be
307 biological (such as proteins) of physical (pH or temperature for example). *B. anthracis* naturally infects
308 humans following spore inhalation, contact with broken skin or ingestion of undercook contaminated meat.
309 These routes present different environmental conditions [2]. It was previously demonstrated that toxin
310 secretion and capsule production could be induced by growing the bacteria in culture media supplemented
311 with HCO_3^- or serum (10-50%) in a CO_2 enriched (5-15%) atmosphere [5, 16, 18, 19, 27-29]. HCO_3^- /CO_2
312 condition were commonly used to study *atxA*, *acpA* and *acpB* regulation and their effect on toxin and
313 capsule biosynthetic genes [16, 18, 30]. Since these conditions always included these two components, it
314 was concluded that *atxA* was induced in response to CO_2, regulating the induction of *acpA* and *acpB*.
315 Although in some reports, capsule production was shown to be AtxA dependent [16, 31], the fact that
316 ΔpXO1 variants are encapsulated contradicts this finding, alluding to additional, AtxA independent
317 regulation of the process [19, 27]. The use of sDMEM as growth media enabled the examination of the
318 effect of CO_2, HCO_3^- and serum on these processes.

319 The parameter of soluble CO_2 is influenced by multiple parameters, such as surface area to volume ratio
320 and aerobic bacterial growth. Therefore, normal growth conditions were set to 100μl media/well (96 well
321 tissue culture plate) for 24h at 37°C under ambient atmosphere (**Figure 1**). This baseline enabled testing
322 the effect of different supplements and/or growth conditions on capsule (**Figure 1**) or toxin (**Figure 2,**
323 **Table 2**) induction. Capsule production is induced by the addition of 10% NRS or growth under a 10% CO_2
324 atmosphere (**Figure 1**). The serum capsule induction (under ambient atmosphere) is *atxA* dependent
325 (**Figure 2**), since there was no significant capsule production in mutants that do not express AtxA
326 (VollumΔ*atxA* and VollumΔpXO1). Alternatively, capsule production in response to CO_2 enriched
327 atmosphere is AtxA independent, as there is no significant difference in capsule production, under these
328 conditions, between AtxA expressing and *atxA* null mutants (**Figures 2, 3**). Toxin secretion, as
329 determined by measuring PA media concentrations, is serum dependent (**Tables 2, 3**), as PA could be
330 detected only in NRS supplemented sDMEM regardless of CO_2 enrichment. Adding HCO_3^- induced toxin
331 secretion in an *atxA* dependent manner, similarly to serum (**Figure 3, Table 3**), with PA expressed by the
332 wild type Vollum and not by the *atxA* null mutant. The same NRS/HCO_3^- dependence and CO_2 independence
333 of PA induction was demonstrated using a previously described mutant strain, in which pXO2 is missing
334 and a copy of the CAP operon was inserted chromosomally, regulated by a PA promotor [17]. This mutant
335 strain produces capsule when grown in sDMEM supplemented with NRS or HCO_3^- but not in non-
336 supplemented sDMEM under 10% CO_2 atmosphere.

337 We also found differences in the speed *B. anthracis* reacts to the different stimuli. This was done by
338 examining toxin secretion and capsule production after a short incubation (5h). HCO_3^- was not as robust
339 as NRS in inducing toxin secretion. Examining PA concertations after 5h incubation in sDMEM
340 supplemented with 0.75% HCO_3^- revealed about 1/10 of the concentration compared to that measured
341 after 24h incubation (**Figure 5, Table 4**). However, NRS induction yielded similar concentrations at these
342 two timepoints. Testing the effect of NRS on capsule production, reveals that 5h incubation under
343 ambient atmosphere, inducing significant PA secretion, does not result in significant capsule production.
344 Growth under a 10% CO_2 atmosphere induced capsule production at 5h even in the absence of
345 supplemented NRS or HCO_3^- (**Table 4**). Hence, Serum seemed more effective then HCO_3^- in inducing toxin
346 secretion, with two process being AtxA dependent. The AtxA independent induction of capsule production
347 by 10% CO_2 appeared more effective then AtxA dependent serum induction (**Figure 5**).
348 Two major regulators; AcpA and AcpB control capsule biosynthesis by promoting transcription of
349 *acpB,C,A,D,E* operon. *acpA* was shown to be regulated by AtxA (activated by NRS), while also depicting
350 an additional *atxA* independent activation capacity by CO_2 and HCO_3^-). This independent activation could
351 be direct or mediated by an as yet unknown, additional promotor, in turn responding to an CO_2 ingredient.
352 We found that deleting *acpA* causes the bacteria to produce significantly less capsule in response to CO_2,
353 while maintaining its ability to respond to NRS or HCO_3^- (**Figure 6**). Deletion of *acpB* did not have any
354 effect on capsule production under all tested conditions (**Figure 6**), supporting our previous *in vivo* data,
355 which showed no effect on virulence [17]. A double deletion of *atxA* and *acpA* or *acpB* revealed that AcpB
356 activity is strictly AtxA dependent under all the conditions tested (**Figure7**). AcpA activity is not
357 affected by the absence of *atxA* in the presence of CO_2 (**Figure7**) but is nulled in response to NRS under
358 ambient atmosphere (**Figure 8**).
359 Our findings delineate the following regulation cascade; CO_2 induces capsule production by activating of
360 *acpA* in an AtxA independent manner. Serum activates the AtxA dependent cascade, inducing toxin
361 secretion and eventually capsule production, by activating *acpA* and *acpB* (**Figure 9**). The order of the
362 processes can be deduced from the lack of capsule production following 5h growth in NRS supplemented
363 sDMEM under ambient atmosphere (**Figure 5**). HCO_3^- induces toxin secretion through the AtxA cascade,
364 but in a less efficient manner (compared to NRS, **Table 4**). Direct activation of capsule production by
365 HCO_3^- in an AtxA independent manner could not be eliminated, since even under ambient atmosphere, it
366 modifies the levels of soluble CO_2 (PCO_2) and possibly induces capsule production via AcpA (**Table 5**). In
367 terms of the initial infection stages of pathology, this differential regulation of toxins and capsule has a
368 logical role. Inhalational and cutaneous infections involve spore phagocytosis by local innate immune cells
369 and their migration to a draining lymph node. While en route (and within the phagocytic cell), the spore

370 needs to germinate and produce the protective capsule. During this stage, toxin production could prove
371 counterproductive, as it disrupts normal cellular function, possibly arresting the cell and preventing it
372 from reaching the lymph node. Once there, toxin production is desirable, possibly enhancing bacterial
373 release from the cells in the lymph node, to the blood stream, promoting pathogenesis. The pathway
374 sensing serum and CO_2 remains to be elucidated and requires more research. Such a pathway may prove
375 common to other pathogens as well as possibly providing additional therapeutic targets for intervention.



376

377 Figure 9. Proposed regulatory scheme for CO_2, Serum and HCO_3^- regulation of capsule production and
378 toxin secretion.

379

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