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

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Abstract

Bacillus anthracis overcome host immune response by producing capsule and secreting toxins. Production of these virulence factors in response to host environment was shown to be regulated by *atxA*, the major virulence regulator, that was shown to be activated by HCO_3^- and CO_2 . While toxin production is regulated directly by *atxA*, capsule production is mediated by either one of two regulators; *acpA* and *acpB*. In addition, it was demonstrated that *acpA* gene have at least two promoters were one of them was linked to *atxA*. We used a genetic approach to study capsule and toxin production under different conditions. Unlike previous works that used NBY- HCO_3^- medium in a CO_2 enriched atmosphere, we used a sDMEM based medium in which toxins and capsule production can be induced in *B. anthracis* 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, while little or no toxins (protective antigen PA) is produced. *atxA* is activated in response to serum in CO_2 independent manner and induce toxins production and capsule production in an *acpA* or *acpB* dependent manner. HCO_3^- activates *atxA* as well, however in non-physiological concentrations. Our findings might be relevant to the first stages of inhalational infection where in the dendritic cell the germinating spore must be protected by the capsule without effecting 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 infect humans via three major routes; lung (inhalation), cutaneous and the gut (digestion) [1, 2]. The infectious form of *B. anthracis* is the spore, that are durable and survive in the environment for decades. In response to the host environment the spore germinates and overcomes the immune system by two major virulence factors; poly- δ -D-glutamic antiphagocytic capsule and the tripartite toxins [3]. The capsule producing genes are encoded as a polycistronic gene cluster on the pXO2 virulence plasmid, *capB,C,A,D,E*. CapB and CapC are the polymerization units while CapA and CapE form the transport channel that exports the capsule polymer from the cytoplasm through the cell wall and membrane to the cell surface [4-6]. On the cell surface CapD hydrolyzes the polymer to shorter chains and covalently link them to the cell wall [7]. The same CapD regulate the length of the bound polymer chains by its hydrolyzation activity [4, 8, 9]. The tripartite toxins are encoded by the pXO1 virulence plasmid, by *pag* (protective antigen-PA), *lef* (lethal factor-LF) and *cya* (edema factor-EF) [1]. LF is a metalloprotease that specifically cleaves members of the MAP kinase regulatory pathway of mammalian cells [10]. EF is a potent calmodulin dependent adenylate cyclase that interferes with cell regulation by elevating the internal cAMP levels [11]. Both LF and EF are driven into the mammalian cells by PA that binds specific receptors ANTXR1 (TEM8) and ANTXR2 (CMG2) [12]. PA binds to the receptor, processed by cell associated furin and oligomerize to form a heptamer that bind LF and EF in a 2:1 ratio [12, 13]. The complex is then phagocytosed and upon lysosome fusion and pH drop the PA complex undergoes a conformational modification the results in LF and EF injection into the cytoplasm. Toxin activity causes, among other, inactivation of immune cells and immune response [12].

The *atxA* was shown to be the major virulence regulator of *B. anthracis* virulent [2, 14]. This pXO1 encoded protein activates a cascade of regulatory processes that results in up and down regulation of chromosomal and plasmid encoded genes [15]. The capsule production is regulated by two pXO2 encoded gene products, *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 Δ pXO1 strains produces capsule [19, 20]. Since *B. anthracis* do not produce toxins and capsule under normal laboratory growth conditions, a specific host simulating growth condition were generated. It was reported that growth of *B. anthracis* in NBY broth supplemented with glucose and bicarbonate in 5-15% CO₂ atmosphere results in toxin secretion and capsule production [16, 20-22]. These growth conditions were used to study different aspects of virulence regulation in *B. anthracis* and these findings indicated that *atxA* is induced in response to HCO₃⁻ and CO₂, activating both toxin and capsule production. This concept implies that toxins and capsule are produced simultaneously in the host.

78 Previously we reported that growth of *B. anthracis* in sDMEM high glucose cell culture medium
 79 supplemented with pyruvate glutamine, nonessential amino acid (referred to in this work as sDMEM) with
 80 addition of 10% serum and incubated in 10%CO₂ atmosphere, induces virulence factor production [17, 19].
 81 Herein we demonstrate that sDMEM can be used to examine the effect of serum (normal rabbit serum -
 82 NRS), HCO₃⁻ and/or CO₂ atmosphere, on the regulation of toxin or capsule production. Using a genetic
 83 approach, we demonstrate that unlike previous reports, *atxA* is induced by NRS or HCO₃⁻ but not CO₂.
 84 The capsule regulator *acpA* was induced by CO₂ in an *atxA* independent manner and by NRS in an *atxA*
 85 dependent manner. The *acpB* was activated only in *atxA* dependent manner when deletion of the later
 86 resulted in loss of *acpB* regulation of capsule production. Our results indicate that the capsule could be
 87 produced independently of the toxins while the toxins will always be co-induced with the capsule.
 88

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, 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). Spores of the different mutants were seeds to a concentration of 5x10⁵ CFU/ml and grown in 96 well tissue culture plate (100μl per well) for 5 or 24h at 37°C in ambient or 10% CO₂ atmosphere.

Mutant strain construction.

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

Toxin quantification

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

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Table 1: Bacterial strains used in this study

Strain	Description/characteristics	Source
<i>B. anthracis</i>		
Vollum	ATCC 14578	IIBR collection
VollumΔpXO1	Complete deletion of the plasmid pXO1	IIBR collection
VollumΔatxA	Complete deletion of the <i>atxA</i> gene	This study
VollumΔacpA	Complete deletion of the <i>acpA</i> gene	[17]
VollumΔacpB	Complete deletion of the <i>acpB</i> gene	[17]
VollumΔacpAΔacpB	Complete deletion of the <i>acpA</i> and <i>acpB</i> genes	[17]
VollumΔpagΔcyaΔlef	Complete deletion of the <i>pag</i> , <i>lef</i> and <i>cya</i> genes	[23]
VollumΔpagΔcyaΔlefΔacpA	Complete deletion of the <i>acpA</i> gene in the VollumΔpagΔcyaΔlef mutant	[17]
VollumΔpagΔcyaΔlefΔacpB	Complete deletion of the <i>acpB</i> gene in the VollumΔpagΔcyaΔlef mutant	[17]
VollumΔpagΔcyaΔlefΔacpAΔacpB	Complete deletion of the <i>acpA</i> and <i>acpB</i> genes in the VollumΔpagΔcyaΔlef mutant	[17]
VollumΔpagΔcyaΔlefΔacpAΔatxA	Complete deletion of the <i>atxA</i> gene in the VollumΔpagΔcyaΔlefΔacpA mutant	This study
VollumΔpagΔcyaΔlefΔacpBΔatxA	Complete deletion of the <i>atxA</i> gene in the VollumΔpagΔcyaΔlefΔacpB mutant	This study
VollumΔpXO2 Δ <i>bclA</i> :: <i>pag_{prom}</i> :: <i>capA-E</i>	Genome insertion of the <i>pagA</i> promoter in front of the CAP operon replacing the <i>bclA</i> gene in the VollumΔpXO2	[17]
VollumΔpXO2 Δ <i>bclA</i> :: <i>pag_{prom}</i> :: <i>capA-E</i> Δ <i>atxA</i>	Deletion of the <i>atxA</i> gene from the strain having genome insertion of the <i>pagA</i> promoter in front of the CAP operon replacing the <i>bclA</i> gene in the VollumΔpXO2	[17]

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Results

The effect of supplements and growth condition on capsule production in sDMEM medium. The basic medium that we used to test capsule and toxins induction in response to different supplements and growth condition was high glucose DMEM that was supplanted with glutamine, pyruvate and non-essential amino acid and is referred to as sDMEM. Since we chose to grow the bacteria in 96 well tissue culture microplate we tested the effect of volume on capsule production. We have inoculated *B. anthracis* Vollum spores (5×10^5 CFU/ml) in 100 μ l, 200 μ l and 300 μ l sDMEM at 37°C in ambient atmosphere for 24h. No capsule production could be detected in 100 μ l sDMEM (Figure 1) but increasing the culture volume to 300 μ l results in capsule production were in 200 μ l only part of the bacteria were encapsulated (Figure 1). This effect could result from dissolved CO₂ generated by bacterial growth and limited gas exchange in the case of low surface to liquid volume. This is supported by the finding that growth in 10% CO₂ atmosphere induces capsule accumulation even in medium volume of 100 μ l sDMEM (Figure 1). Normal rabbit serum (NRS) induces capsule accumulation regardless of atmosphere composition, since all the bacteria were encapsulated in ambient or 10% CO₂ atmosphere (Fig 1). Based on these experiments we used 100 μ l sDMEM medium in 96 well tissue-culture plates for all of our induction experiments.

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144 **Figure 1. The effect of growth conditions on capsule accumulation by *B. anthracis* Vollum.** Spores
 145 were seeded into 100μl, 200μl or 300μl of DMEM and incubated at 37°C in an ambient atmosphere (upper panel) for
 146 24h. To examine the effect of serum or CO₂ on capsule production, spores were seeded into 100μl DMEM as is or
 147 supplemented with 10% NRS and incubated at 37°C in an ambient or 10% CO₂ atmosphere (as indicated on the right)
 148 for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright
 149 outer layer).

150 **The role of *atxA* on capsule production and toxin secretion under different growth conditions.** To
 151 test the role of *atxA* on capsule and toxins production following growth in sDMEM under the different
 152 growth conditions we compared the wild type Vollum strain to the ΔpXO1 or Δ*atxA* mutants. None of the

153 strains produced capsule under ambient atmosphere in the un-supplemented sDMEM medium (Figure 2).
 154 Addition of 10% NRS resulted in capsule production by the wild type Vollum strain but not by the *atxA*
 155 null mutants ($\Delta pXO1$ or $\Delta atxA$ Figure 2). In a 10% CO_2 atmosphere all the strains were encapsulated
 156 regardless of the presence or absence of *atxA*. Hence, the effect of NRS in the presence of CO_2 on
 157 capsule production under these conditions could not be resolved since capsule production is induced in
 158 response to the presence of CO_2 or NRS. Since it appeared that the response to NRS is *atxA* dependent
 159 we can assume that capsule production in the *atxA* null mutants derived from only the CO_2 (Figure 2).

160
 161 Figure 2. Capsule production of the Vollum wild type, $\Delta pXO1$ or $\Delta atxA$ mutants under different growth
 162 conditions. The effect of the absence of *pXO1* or the *atxA* gene on capsule accumulation under the different growth

conditions, Spores of the wild type and different mutants (top panel) were seeded into 100µl of DMEM as is or supplemented with 10% NRS and incubated at 37°C in an ambient or 10% CO₂ atmosphere (as indicated on the right) for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright outer layer).

As *atxA*, the major virulence regulator is regulating the toxins expression (LF, EF and PA), we determined the concentration in the growth medium of the Vollum and VollumΔ*atxA* mutant (in Δ*pXO1* all the tox genes are missing). Toxins secretion was determined by ELISA of the most abundant component, the PA. PA was detected only when the growth medium was supplemented with NRS (Table 1). According to our results (Table 1), CO₂ enriched atmosphere reduced the PA levels to about 30%, reduction that was maintained through different independent experiments. However, we cannot eliminate the possibility that this difference results from differences in growth rates under these conditions. No PA secretion could be detected in the *atxA* null mutant under any of the tested growth conditions.

The fact that we find that CO₂ atmosphere by itself do not activate *atxA* dependent toxin induction, is surprising since it was repeatedly demonstrated that *atxA* is regulated by HCO₃⁻. Therefore, we tested capsule production and toxin secretion in a sDMEM supplemented with 0.75% HCO₃⁻ in 10% CO₂ atmosphere. The CO₂ atmosphere was chosen since the addition of HCO₂⁻ dramatically increase the levels of the soluble CO₂ in the medium even under ambient conditions and we were interested to maintain the controls in as close conditions as possible.

Table 1. Protective Antigen secretion of Vollum or VollumΔ*atxA* under different growth conditions.

	VollumΔ <i>atxA</i>	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

185 **Figure 3. Effect of 0.75% HCO₃⁻ on capsule accumulation of *Vollum*, *Vollum*ΔatxA or *Vollum*ΔpXO1. Spores**
186 **were seeded into 100μl of DMEM as is or supplemented with 0.75% HCO₃⁻ or 10%NRS and incubated at 37°C in an**
187 **atmosphere of 10% CO₂ for 24h. The presence or absence of capsule were determined by India ink negative staining**
188 **(capsule seen as a bright outer layer). The different mutations are indicated (top panel).**

189

190 **Table 2. PA secretion by *Vollum* and *Vollum*ΔatxA in medium supplemented with HCO₃⁻**

	<i>Vollum</i> ΔatxA	<i>Vollum</i>	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

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192 As we demonstrated capsule accumulation is CO₂ dependent, therefore all the strains are encapsulated
193 under all the condition tested (**Figure 3**). PA secretion was tested only in the growth medium of *Vollum*
194 and *Vollum*ΔatxA. Supplementing the sDMEM with 0.75% HCO₃⁻ induced PA secretion by the *Vollum* strain
195 but not by the *Vollum*ΔatxA mutant, indicating that HCO₃⁻ induce toxin secretion in an atxA dependent
196 manner (**Table 2**). To validate these findings, we used a previously reported *Vollum*ΔpXO2 chimera in

197 which we substituted the genomic *bclA* gene with a PA_{prom} regulated CAP operon. In this case capsule
198 accumulation is an indication for *atxA* dependent activation of the PA promotor that activates the CAP
199 genes.

200 **Figure 4. Capsule accumulation in response to HCO₃⁻ in a PA_{prom} - regulated genome cluster of CAP operon.**
201 Spores were seeded into 100μl of DMEM as is or supplemented with 0.75% HCO₃⁻ or 10%NRS and incubated at 37°C
202 in an atmosphere of 10% CO₂ for 24h. The presence or absence of capsule were determined by India ink negative
203 staining (capsule seen as a bright outer layer). The different mutations are indicated (top panel).

204
205 While 10% CO₂ atmosphere induce capsule production in the Vollum wild type strain little or no capsule
206 production could be detected in the Vollum $\Delta pXO2$ chimeric strain (**Figure 4**). Supplementing the media
207 with 0.75% HCO₃⁻ to the growth medium results in capsule accumulation in the Vollum and Vollum $\Delta pXO2$
208 chimera, however deletion of the *atxA* resulted in capsule loss of the Vollum $\Delta pXO2$ chimeric strain (**Figure**
209 **4**). Same results were obtained by supplementing the medium with 10% NRS. These results support the
210 PA secretion experiment that indicate that HCO₃⁻ and NRS are inducing toxin secretion in an *atxA*
211 dependent manner.

The effect of short incubation on capsule and toxins accumulation in response the different growth conditions. Aerobic growth affects different parameters of the liquid medium such as pH and O₂/CO₂ concentrations especially when the bacteria reaches high concentration (CFU/ml). As was shown previously, capsule production and toxins secretion can be detected following 2- 5h of growth in sDMEM in 10% CO₂ atmosphere. To eliminate as much as possible the changes in media condition as result of bacterial growth we examine capsule production and PA secretion following 5h growth in different growth conditions. Growth of the Vollum strain in ambient condition did not result in any capsule accumulation or toxin secretion following 24h incubation (**Figure 2, Table 1**) or a short 5h incubation (**Figure 5, Table 3**). Supplementing the media with 10% NRS induced capsule production and PA secretion flowing 24h incubation in ambient atmosphere (**Figure 2, Table 1**). Examination these parameters following a shorter, 5h incubation, demonstrating PA accumulation (**Table 3**) but no capsule accumulation (**Figure 5**). This PA accumulation is AtxA dependent as deletion of the *atxA* gene resulted in no PA accumulation following 5h (**Table 3**) or 24h incubation (**Table 1**). Incubating the bacteria in 10% CO₂ atmosphere for 5h result in capsule accumulation (**Figure 5**) the same as was observed following a 24h incubation (**Figures 1-3**). This capsule accumulation was *atxA* independent and was not dramatically affected by the addition of 10%NRS or HCO₃⁻ (**Figure 5**). PA secretion was not induced by 10%CO₂ per se buy required addition of NRS or HCO₃⁻ (**Table 3**), however unlike the 24h incubation (**Table 2**), the PA that was secreted in respond to HCO₃⁻ was less than 10% of that induced by NRS (**Table 3**). Under all conditions the PA secretion was AtxA dependent.

Figure 5. Effect of the absence of *atxA* gene on capsule accumulation after short (5h) growth under the different growth conditions. Spores of the wild type and $\Delta atxA$ mutant (top panel) were seeded into 100μl of DMEM as is or supplemented with 10% NRS and incubated at 37°C in an ambient or 10% CO₂ atmosphere (as indicated

on the right) for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright outer layer).

Table 3. PA secretion by Vollum and Vollum Δ atxA flowing 5h growth in different conditions

	Vollum Δ atxA	Vollum	Atmosphere	Supplements
PA μ g/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 ⁻

Regulation of *acpA* and *acpB* in response to different growth conditions. As toxin production is induced in an *atxA* dependent manner in response to HCO₃⁻ or NRS, capsule production is also induced by CO₂ enriched (10%) atmosphere. Since capsule production is regulated by two regulatory proteins, AcpA and AcpB, we tested the effect of the deletion of each on of these genes on capsule accumulation in response to the different growth conditions. The complete coding region of *acpA* or *acpB* was deleted independently in the wild type Vollum or the toxin deficient mutant Vollum Δ Tox (ref). As was previously shown for the wide type Vollum strain (Figure 2) none of these mutants accumulate capsule flowing growth in sDMEM with ambient atmosphere (Figure 6). The presence of *acpA* or *acpB* is sufficient for capsule accumulation in sDMEM supplemented with 10% NRS, regardless to the presence or absence of 10% CO₂ atmosphere (Figure 6). However, in the absence of NRS, only AcpA expressing mutants accumulate significant capsule when grown in 10% CO₂ atmosphere. 0.75% HCO₃⁻ induced capsule accumulation in the presence of *acpA* or *acpB*. Mutants deleted of *acpA* (expressing only *acpB*), did not accumulate significant capsule in 10% CO₂ atmosphere. To examine the role of *atxA* in these processes, we deleted the *atxA* gene in our Vollum Δ Tox Δ *acpA* or Δ *acpB* mutants. Deleting the Δ *atxA* in the Vollum Δ Tox Δ *acpA*, which express only the *acpB*, abolishes capsule accumulation under all tested conditions (Figure 7). However, deleting the *atxA* gene in the Vollum Δ Tox Δ *acpB*, which express only *acpA* did not affect capsule accumulation, relative to the *atxA* expressing mutant, indicating that *acpA* operates in an *atxA* independent manner (Figure 7).

As we demonstrated (Figure 2), capsule accumulation could be induced in ambient atmosphere by adding 10% NRS to the growth media. This induction is AtpA dependent since no capsule accumulation was detected in the Vollum Δ atxA mutant under these conditions (Figure 2). Since AcpA dependent capsule accumulation in 10% CO₂ atmosphere was AtpA independent, we tested the role of AtpA on AcpA dependent capsule accumulation in response to 10% NRS in ambient atmosphere. As 10% NRS induces capsule accumulation of Vollum Δ acpB in ambient atmosphere (Figure 6, Figure 8) we tested the effect

of *atxA* deletion on capsule accumulation under these conditions. Unlike the CO_2 induction, in ambient atmosphere, AcpA dependent capsule accumulation in response to NRS is AtxA dependent (Figure 8).

Figure 6. The effect of absence of *acpA* or *acpB* on capsule accumulation in response to 10% NRS in an ambient or 10% CO_2 atmosphere. Spores of the Δ acpA or *acpB* mutants (top panel) were seeded into 100 μ l of DMEM as is or supplemented with 0.75% HCO_3^- or 10% NRS and incubated at 37°C in an ambient or 10% CO_2 atmosphere (as indicated on the right) for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright outer layer).

Figure 7. Effect of *AtxA* on Capsule accumulation in the presence of only *AcpA* or *AcpB*. Spores of the different mutants (top panel) were seeded into 100μl of DMEM as is or supplemented with 0.75% HCO₃⁻ or 10% NRS and incubated at 37°C in 10% CO₂ atmosphere (as indicated on the right) for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright outer layer).

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Figure 8. Effect of *AtxA* on Capsule accumulation in response to 10% NRS in ambient atmosphere. Spores of the different mutants (top panel) were seeded into 100μl of DMEM as is or supplemented with 10% NRS and

284 incubated at 37°C in ambient atmosphere (as indicated on the right) for 24h. The presence or absence of capsule
285 were determined by India ink negative staining (capsule seen as a bright outer layer).

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Discussion

For successful invasion the pathogen must regulate its virulence factors in a way that will maximize their effect on the host defense mechanisms. The trigger of such activation is usually host derived and can be biological (such as proteins) or physical (pH or temperature for example). *B. anthracis* naturally infect humans following spore inhalation, contact with broken skin or ingestion of undercooked contaminated meat, in each of them the bacteria faces different environmental conditions [2]. It was previously demonstrated that toxins secretion and capsule accumulation could be induced by growing the bacteria in culture media supplemented with HCO_3^- or serum (10-50%) in a CO_2 enriched (5-15%) atmosphere [5, 16, 18, 19, 26-28]. $\text{HCO}_3^- / \text{CO}_2$ condition were used to study in most studies of *atxA*, *acpA* and *acpB* regulation and their effect on toxins and capsule biosynthetic genes [16, 18, 29]. Since these conditions always included these two components it was concluded that *atxA* was induced in response to CO_2 and regulate the induction of *acpA* and *acpB*. Although in some reports, capsule accumulation was shown to be *atxA* dependent [16, 30], the fact that ΔpXO1 variants are encapsulated contradict this finding, indicating additional *atxA* independent regulation of the process [19, 26]. The use of sDMEM as a growth media enabled the examination of the effect of CO_2 , HCO_3^- and serum on these processes.

The parameter of soluble CO_2 is influenced by multiple parameters such as surface area to volume ratio and aerobic bacterial growth. Therefore, normal growth conditions were determined as 100 μl media/well of a 96 well tissue culture plate for 24h at 37°C in ambient condition (Figure 1). This baseline enabled testing of the different supplements and/or growth conditions on capsule (Figure 1) or toxin (Figure 2, Table 1). Capsule production is induced by the addition of 10% NRS or growth in a 10% CO_2 atmosphere (Figure 1). The serum capsule induction (in ambient atmosphere) is *atxA* dependent (Figure 2) since there was no significant capsule accumulation in the mutants that did not express *AtxA* (Vollum ΔatxA and Vollum ΔpXO1). Alternatively, capsule accumulation in response to CO_2 enriched atmosphere is *atxA* independent, as there is no significant difference in capsule accumulation under these conditions between *AtxA* expressing and *atxA* null mutants (Figures 2, 3). Toxin secretion, as determined by PA media concentration is serum dependent (Tables 1, 2), as PA could be detected only in NRS supplemented sDMEM regardless of the CO_2 enriched atmosphere. HCO_3^- as serum induced toxin secretion in an *atxA* dependent manner (Figure 3, Table 2) where PA accumulated in the wild type Vollum and not in the *atxA* null mutant. The same NRS/ HCO_2^- dependence and CO_2 independence of *pag* (PA) induction was demonstrated using a previously reported mutant strain which is missing *pXO2* and have a chromosomal copy of the *CAP* operon regulated by a *pag* promoter [17]. This mutant strain accumulates capsule when grown in sDMEM that was supplemented with NRS or HCO_3^- but not in sDMEM in 10% CO_2 atmosphere.

We found differences in rates that *B. anthracis* reacts to the different stimulants. Examining the toxins secretion and capsule accumulation after short incubation of 5h. HCO_3^- was not as robust as NRS in inducing toxins secretion. Examining the PA concentration after 5h incubation in DMEM supplemented with 0.75% HCO_3^- revealed about 1/10 of the concentration after a 24h incubation (Figure 5, Table 3), were with NRS the concentration in these two timepoints was similar. Testing the effect of serum on capsule accumulation reveals that 5h incubation in ambient atmosphere which result in significant PA secretion, did not result in significant capsule accumulation. 5h growth in a 10% CO_2 atmosphere induced capsule accumulation even in the absence of supplemented NRS or HCO_3^- (Table 3). Hence, Serum seemed more effective than HCO_3^- in inducing toxin secretion, two process that are *atxA* dependent. The *atxA* independent 10% CO_2 atmosphere appeared more effective than *atxA* dependent serum for capsule accumulation (Figure 5).

Two major regulators; AcpA and AcpB control capsule biosynthesis by promoting transcription of *acpB,C,A,D,E* operon. *acpA* was shown to be regulated by *atxA* (assumed by CO_2 and HCO_3^-) and to possess an additional *atxA* independent promoter. We found that by deleting *acpA* the mutant accumulates significantly less capsule in response to CO_2 but maintain its ability to respond to NRS or HCO_3^- (Figure 6). Deletion of *acpB* did not have any effect on capsule accumulation under all tested conditions (Figure 6) which support our previous in vivo data. Deletion of both *atxA* and *acpA* or *acpB* revealed that AcpB activity is strictly *AtxA* dependent under all the conditions tested (Figure 7). AcpA activity is not affected by the absence of *atxA* in the presence of CO_2 , (Figure 7) but is nulled in response to NRS in an ambient environment (Figure 8).

Our findings support the following regulation cascade; CO_2 is inducing capsule accumulation via the activation of *acpA* in an *AtxA* independent manner. Serum activate the *AtxA* dependent cascade that induce toxins secretion and eventually capsule accumulation by activating *acpA* and *acpB* (Figure 9) since there was no capsule accumulation following 5h growth in NRS containing in ambient atmosphere (Figure 5). HCO_3^- induces toxin secretion by *AtxA* cascade, in a less efficient manner (relative to NRS, Table 3). Direct activation of capsule accumulation by HCO_3^- in *AtxA* independent manner could not be eliminated since even in ambient atmosphere, it modifies the levels of soluble CO_2 (PCO_2) and possibly induces capsule production via *acpA*. Theoretically, this differential regulation of toxins and capsule have logical role in *B. anthracis* pathogenicity. Inhalational and cutaneous infection involve spore engulfing and migration to a draining lymph node. Within the phagocytic (dendritic) cell the spore germinates and produce capsule that protect the bacteria from phagolysis. Toxin production in this stage is counterproductive as it could result in cell arrest that might interfere with the pathogenic pathway. Toxin production may enhance

353 once the bacteria is released from the cell in the lymph node. The serum and CO₂ sensing pathway is still
354 to be determined and might be common to other pathogens and therefore be a target for therapeutics.

355 Figure 9. Proposed regulation of CO₂, Serum and HCO₃⁻ on capsule production and toxin secretion.

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