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 promotors 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 were 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 calmudolin 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 phagocyted 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 cases, among other, inactivation of immune cells and immune response [12].

The *atxA* was shown to be the major virulence regular 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 ΔpXO1 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% CO_2 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* in induced in response to HCO_3^- and CO_2, activating both toxin and capsule production. This concept implies that toxins and capsule are produced simultaneously in the host.

Previously we reported that growth of *B. anthracis* in sDMEM high glucose cell culture medium supplemented with pyruvate glutamine, nonessential amino acid (referred to in this work as sDMEM) with addition of 10% serum and incubated in 10%CO_2 atmosphere, induces virulence factor production [17, 19]. Herein we demonstrate that sDMEM can be used to examine the effect of serum (normal rabbit serum – NRS), HCO_3^- and/or CO_2 atmosphere, on the regulation of toxin or capsule production. Using a genetic approach, we demonstrate that unlike previous reports, *atxA* is induced by NRS or HCO_3^- but not CO_2. The capsule regulator *acpA* was induced by CO_2 in an *atxA* independent manner and by NRS in an *atxA* dependent manner. The *acpB* was activated only in *atxA* dependent manner when deletion of the later resulted in loss of *acpB* regulation of capsule production. Our results indicate that the capsule could be produced independently of the toxins while the toxins will always be co-induced with the capsule.

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 wail) for 5 or 24h at 37oC 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 determine by capture ELISA using the combination of a polyclonal and monoclonal αPA antibodies as previously described [23]

125 **Table 1: Bacterial strains used in this study**

Strain	Description/characteristics	Source
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*	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*	Genome 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 genome insertion of the *pagA* promotor in front of the CAP operon replacing the *bclA* 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 ($5x10^5$ CFU/ml) in 100μl, 200μl and 300μl sDMEM at 37°C in ambient atmosphere for 24h. No capsule production could be detected in 100μl sDMEM (**Figure 1**) but increasing the culture volume to 300μl results in capsule production were in 200μl only part of the bacteria were encapsulated (**Figure 1**). This effect could result from dissolved CO_2 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% CO_2 atmosphere induces capsule accumulation even in medium volume of 100μl sDMEM (**Figure 1**). Normal rabbit serum (NRS) induces capsule accumulation regardless of atmosphere composition, since all the bacteria were encapsulated in ambient or 10% CO_2 atmosphere (Fig 1). Based on these experiments we used 100μl sDMEM medium in 96 well tissue-culture plates for all of our induction experiments.

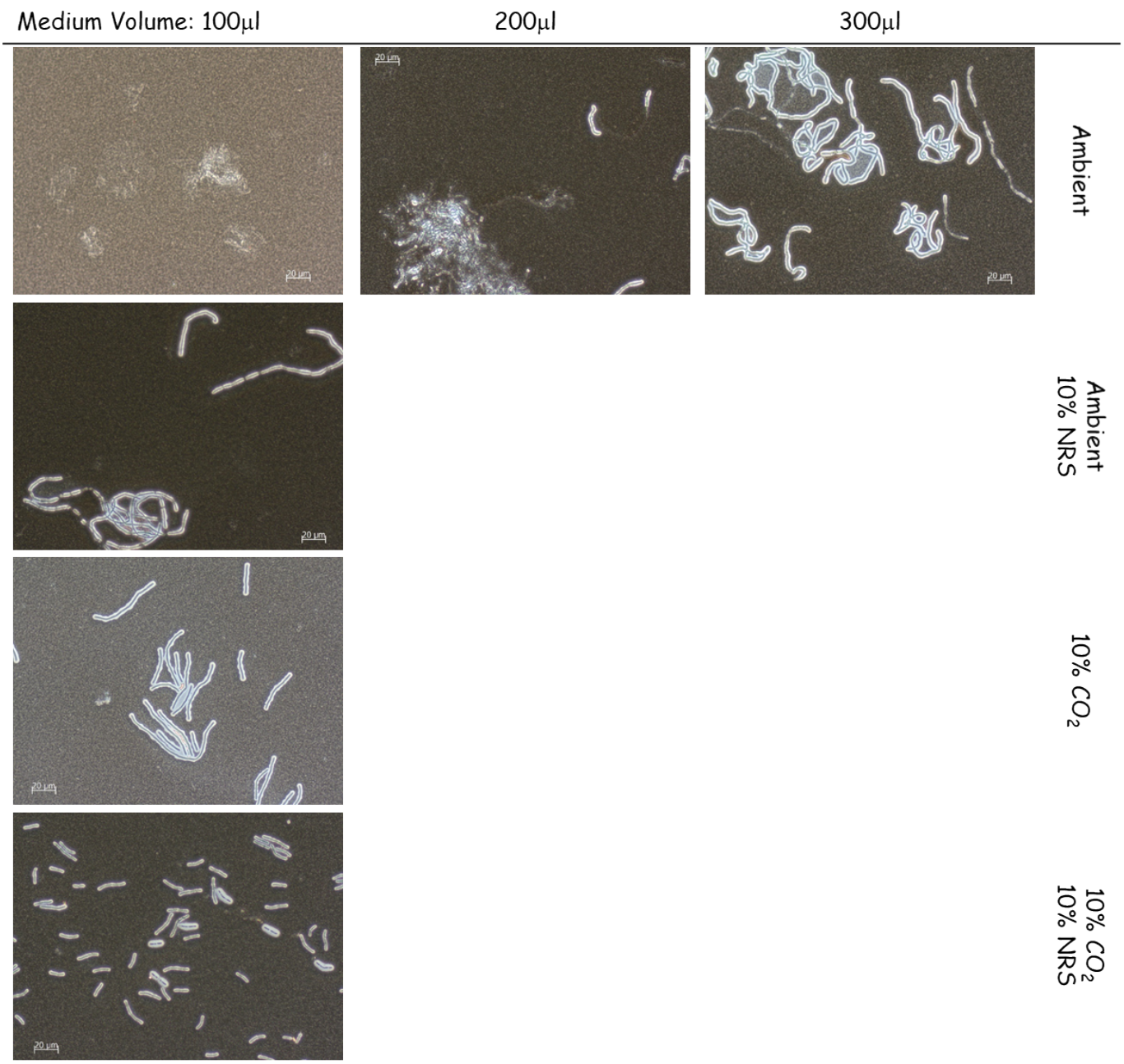
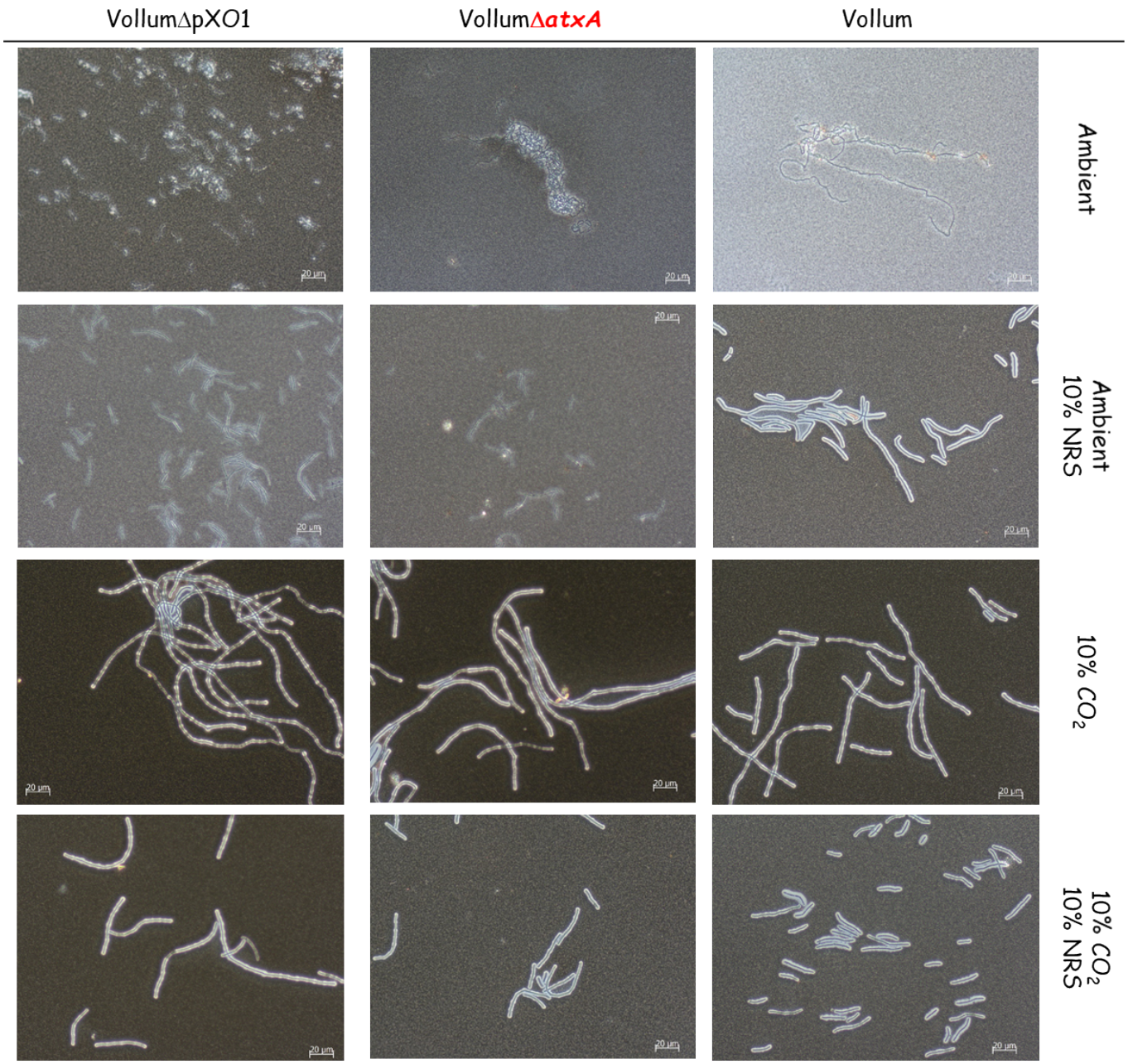


Figure 1. The effect of growth conditions on capsule accumulation by *B. anthracis* Vollum. Spores were seeded into 100µl, 200µl or 300µl of DMEM and incubated at 37°C in an ambient atmosphere (upper panel) for 24h. To examine the effect of serum or CO_2 on capsule production, spores were seeded into 100µl DMEM as is or supplemented with 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).

The role of *atxA* on capsule production and toxin secretion under different growth conditions. To test the role of *atxA* on capsule and toxins production following growth in sDMEM under the different 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 (ΔpXO1 or Δ*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 form only the CO_2 (**Figure 2**).



160

161 Figure 2. Capsule production of the Vollum wild type, ΔpXO1 or Δ*xtXA* 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% 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).

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 Vollum∆*atxA* mutant (in ∆pXO1 al 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**), CO_2 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 CO_2 atmosphere by itself do not activate *atxA* dependent toxin induction, is surprising since it was repeatedly demonstrated that *atxA* is regulated by HCO_3^-. Therefore, we tested capsule production and toxin secretion in a sDMEM supplemented with 0.75% HCO_3^- in 10% CO_2 atmosphere. The CO_2 atmosphere was chosen since the addition of HCO_2^- dramatically increase the levels of the soluble CO_2 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 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

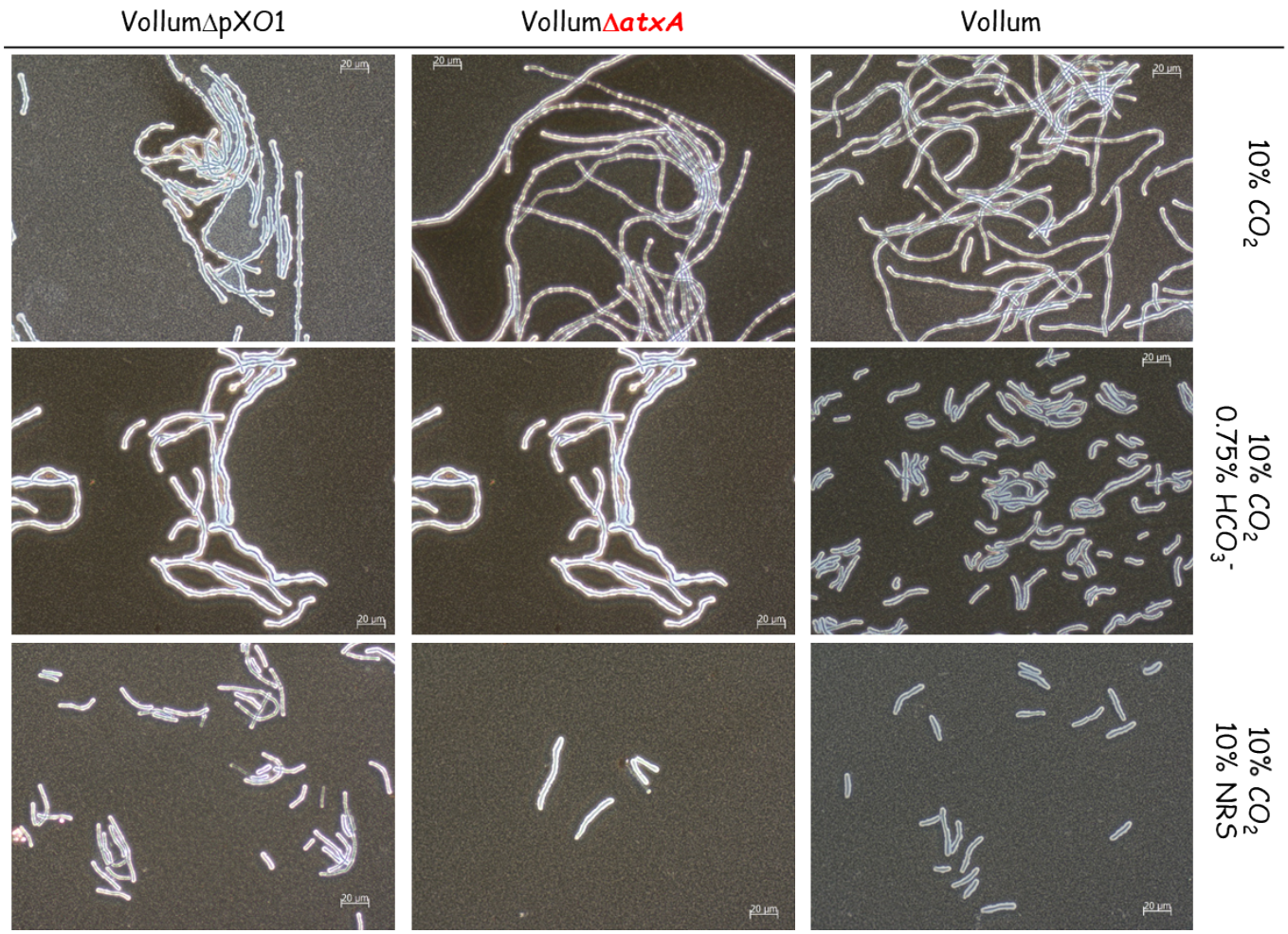


Figure 3. Effect of 0.75% HCO_3^- on capsule accumulation of Vollum, VollumΔ*atxA* or VollumΔpXO1. Spores were seeded into 100µl of DMEM as is or supplemented with 0.75% HCO_3^- or 10%NRS and incubated at 37°C in an atmosphere of 10% CO_2 for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule seen as a bright outer layer). The different mutations are indicated (top panel).

Table 2. PA secretion by Vollum and VollumΔ*atxA* in medium 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

As we demonstrated capsule accumulation is CO_2 dependent, therefore all the strains are encapsulated under all the condition tested (**Figure 3**). PA secretion was tested only in the growth medium of Vollum and VollumΔ*atxA*. Supplementing the sDMEM with 0.75% HCO_3^- induced PA secretion by the Vollum strain but not by the VollumΔ*atxA* mutant, indicating that HCO_3^- induce toxin secretion in an *atxA* dependent manner (**Table 2**). To validate these findings, we used a previously reported VollumΔpXO2 chimera in

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

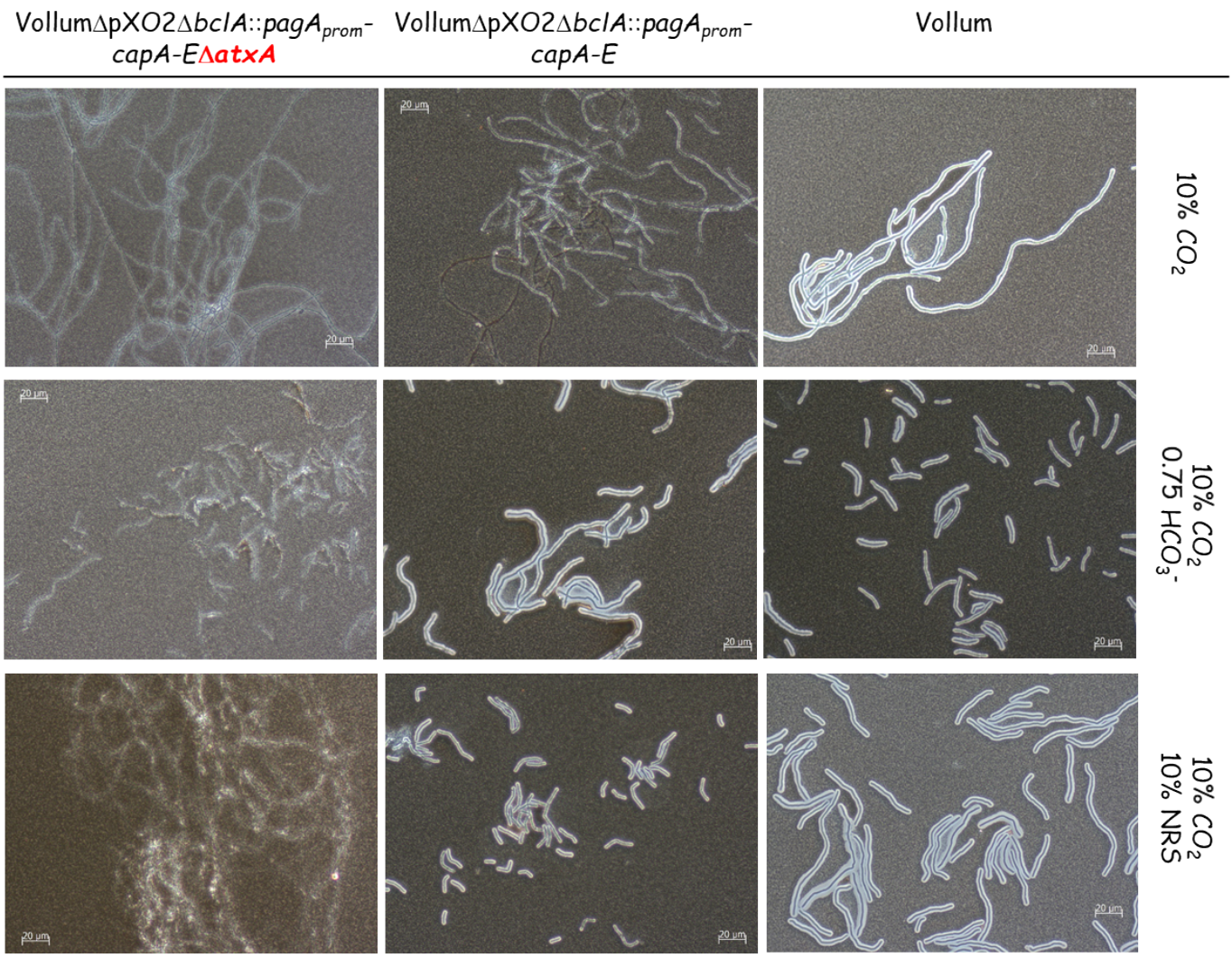
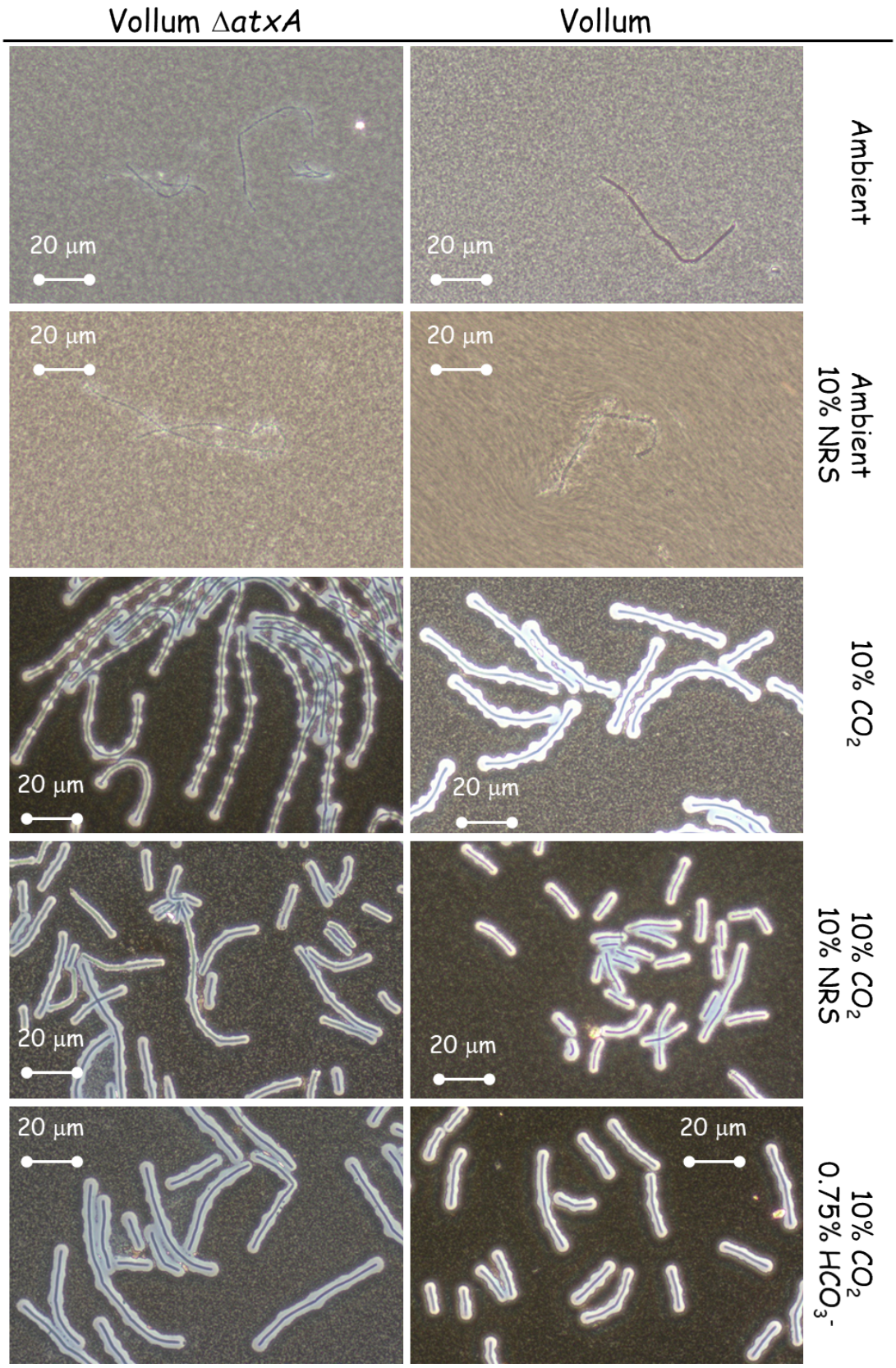


Figure 4. Capsule accumulation in response to HCO_3^- in a PA_{prom}- regulated genome cluster of CAP operon. Spores were seeded into 100µl of DMEM as is or supplemented with 0.75% HCO_3^- or 10%NRS and incubated at 37°C in an atmosphere of 10% CO_2 for 24h. The presence or absence of capsule were determined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

While 10% CO_2 atmosphere induce capsule production in the Vollum wild type strain little or no capsule production could be detected in the VollumΔpXO2 chimeric strain (**Figure 4**). Supplementing the media with 0.75% HCO_3^- to the growth medium results in capsule accumulation in the Vollum and VollumΔpXO2 chimera, however deletion if the *atxA* resulted in capsule loss of the VollumΔpXO2 chimeric strain (**Figure 4**). Same results were obtained by supplementing the medium with 10% NRS. These results support the PA secretion experiment that indicate that HCO_3^- and NRS are inducing toxin secretion in an *atxA* 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 O_2/CO_2 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% CO_2 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% CO_2 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 HCO_3^- (**Figure 5**). PA secretion was not induced by 10%CO_2 per se buy required addition of NRS or HCO_3^- (**Table 3**), however unlike the 24h incubation (**Table 2**), the PA that was secreted in respond to HCO_3^- was less than 10% of that induced by NRS (**Table 3**). Under all conditions the PA secretion was AtxA dependent.



236 Figure 5. Effect of the absence of *atxA* gene on capsule accumulation after short (5h) growth under the
237 different growth conditions. Spores of the wild type and Δ*atxA* mutant (top panel) were seeded into 100μl of
238 DMEM as is or supplemented with 10% NRS and incubated at 37°C in an ambient or 10% CO_2 atmosphere (as indicated

239 on the right) for 24h. The presence or absence of capsule were determined by India ink negative staining (capsule
240 seen as a bright outer layer).
241
242 Table 3. PA secretion by Vollum and VollumΔ*atxA* flowing 5h growth in different conditions

	VollumΔ*atxA*	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-

243
244 **Regulation of *acpA* and *acpB* in response to different growth conditions.** As toxin production is induced
245 in an *atxA* dependent manner in response to HCO_3^- or NRS, capsule production is also induced by CO_2
246 enriched (10%) atmosphere. Since capsule production is regulated by two regulatory proteins, AcpA and
247 AcpB, we tested the effect of the deletion of each on of these genes on capsule accumulation in response
248 to the different growth conditions. The complete coding region of *acpA* or *acpB* was deleted
249 independently in the wild type Vollum or the toxin deficient mutant VollumΔTox (ref). As was previously
250 shown for the wide type Vollum strain (**Figure 2**) none of these mutants accumulate capsule flowing growth
251 in sDMEM with ambient atmosphere (**Figure 6**). The presence of *acpA* or *acpB* is sufficient for capsule
252 accumulation in sDMEM supplemented with 10% NRS, regardless to the presence or absence of 10% CO_2
253 atmosphere (**Figure 6**). However, in the absence of NRS, only AcpA expressing mutants accumulate
254 significant capsule when grown in 10% CO_2 atmosphere. 0.75% HCO_3^- induced capsule accumulation in the
255 presence of *acpA* or *acpB*. Mutants deleted of *acpA* (expressing only *acpB*), did not accumulate significant
256 capsule in 10% CO_2 atmosphere. To examine the role of *atxA* in these processes, we deleted the *atxA*
257 gene in our VollumΔToxΔ*acpA* or Δ*acpB* mutants. Deleting the Δ*atxA* in the VollumΔToxΔ*acpA*, which
258 express only the *acpB*, abolishes capsule accumulation under all tested conditions (**Figure 7**). However,
259 deleting the *atxA* gene in the VollumΔToxΔ*acpB*, which express only *acpA* did not affect capsule
260 accumulation, relative to the *atxA* expressing mutant, indicating that *acpA* operates in an *atxA*
261 independent manner (**Figure 7**).
262 As we demonstrated (**Figure 2**), capsule accumulation could be induced in ambient atmosphere by adding
263 10% NRS to the growth media. This induction is AtxA dependent since no capsule accumulation was
264 detected in the VollumΔ*atxA* mutant under these conditions (**Figure 2**). Since AcpA dependent capsule
265 accumulation in 10% CO_2 atmosphere was AtxA independent, we tested the role of AtxA on AcpA
266 dependent capsule accumulation in response to 10% NRS in ambient atmosphere. As 10% NRS induces
267 capsule accumulation of VollumΔ*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**).

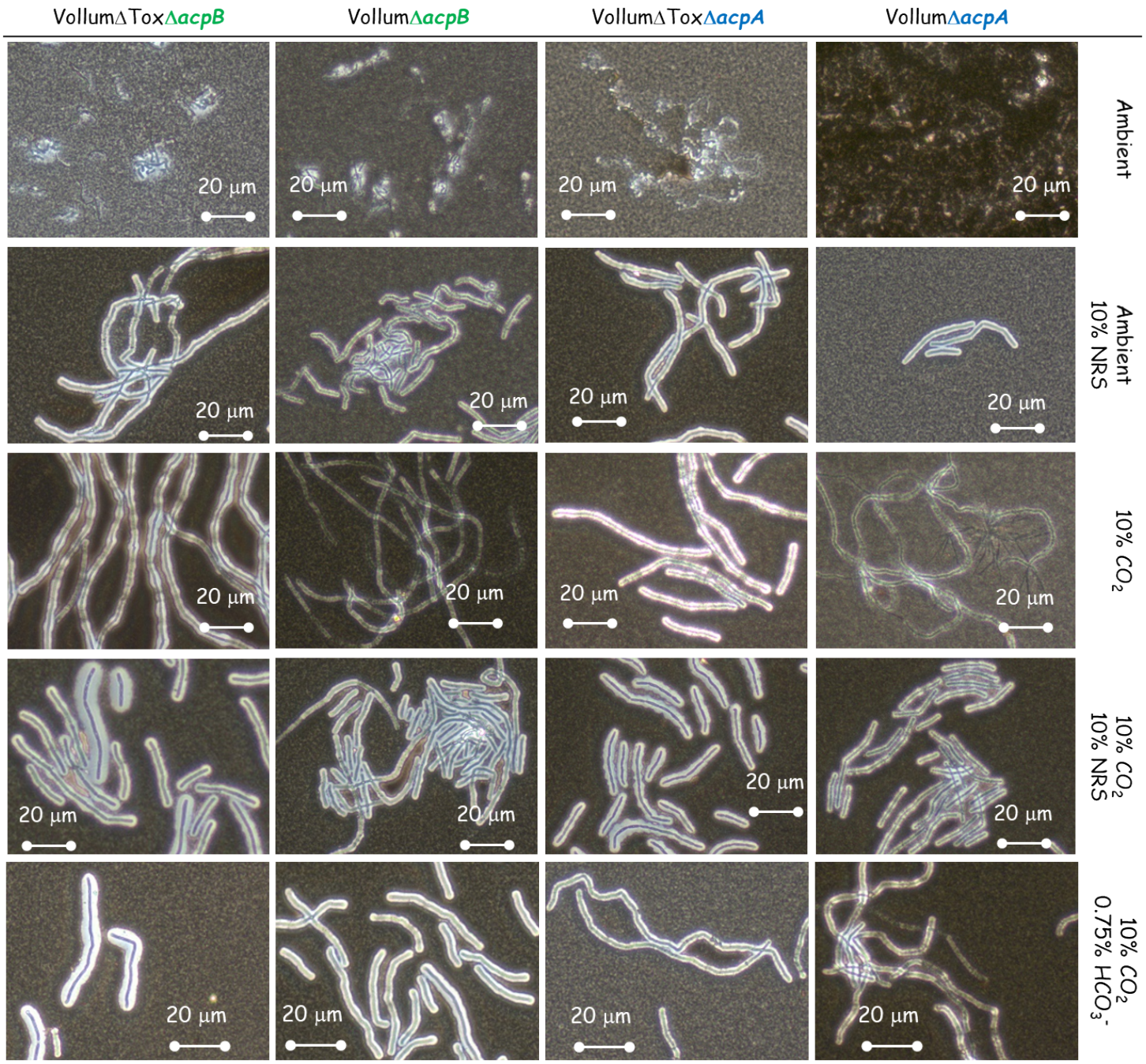
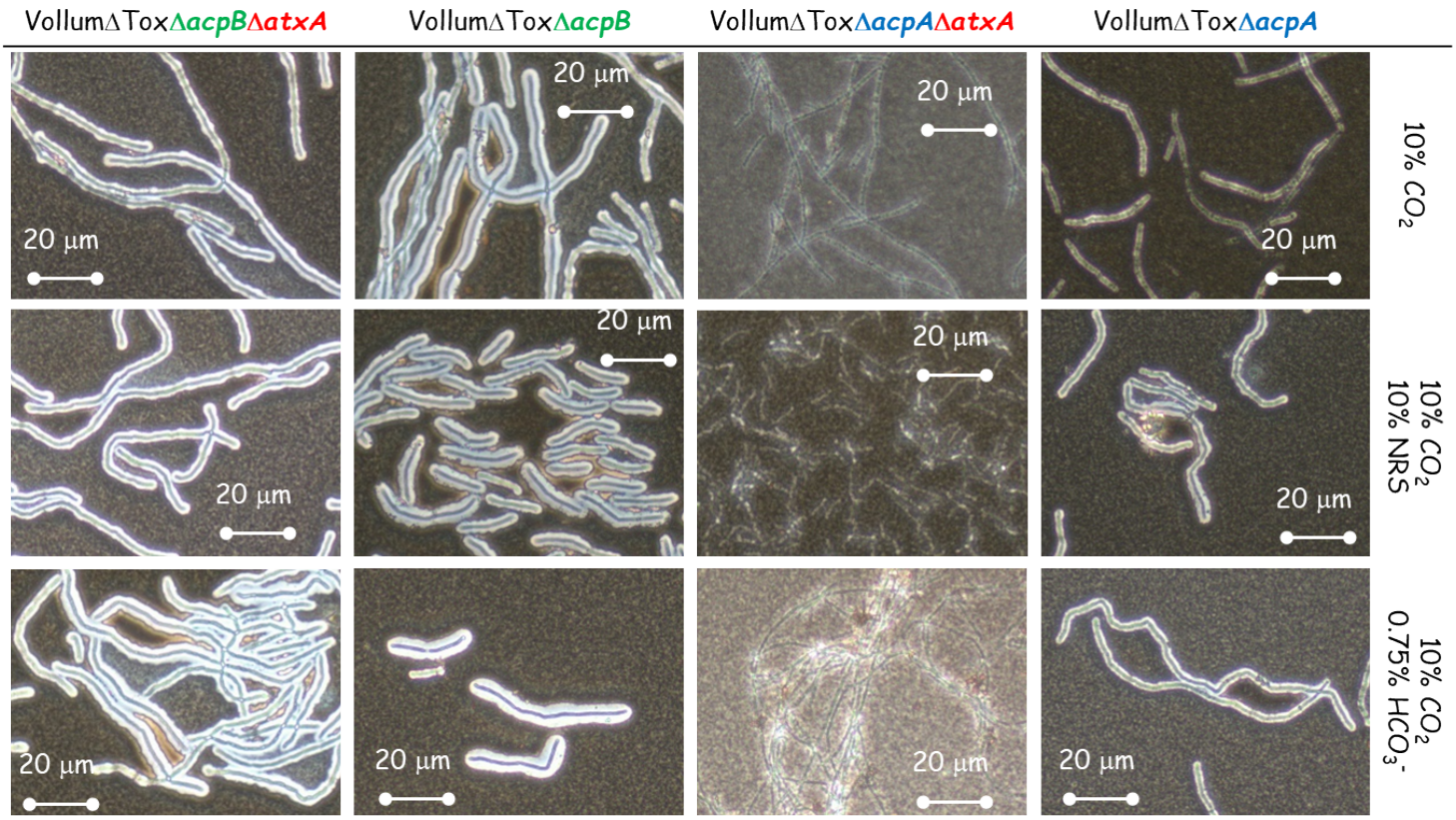


Figure 6. The effect of absence of *acpA* or *acpB* on capsule accumulation in response to 10% NRS in an ambient or 10% CO_2 atmosphere. Spores of the Δ*acpA* or *acpB* mutants (top panel) were seeded into 100μl of DMEM as is or supplemented with0.75% HCO_3^- or 10% NRS and incubated at 37°C in an ambient 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).



277 Figure 7. Effect of AtxA on Capsule accumulation in the presence of only AcpA or AcpB. Spores of the
278 different mutants (top panel) were seeded into 100µl of DMEM as is or supplemented with 0.75% HCO_3^- or 10% NRS
279 and incubated at 37°C in 10% CO_2 atmosphere (as indicated on the right) for 24h. The presence or absence of capsule
280 were determined by India ink negative staining (capsule seen as a bright outer layer).
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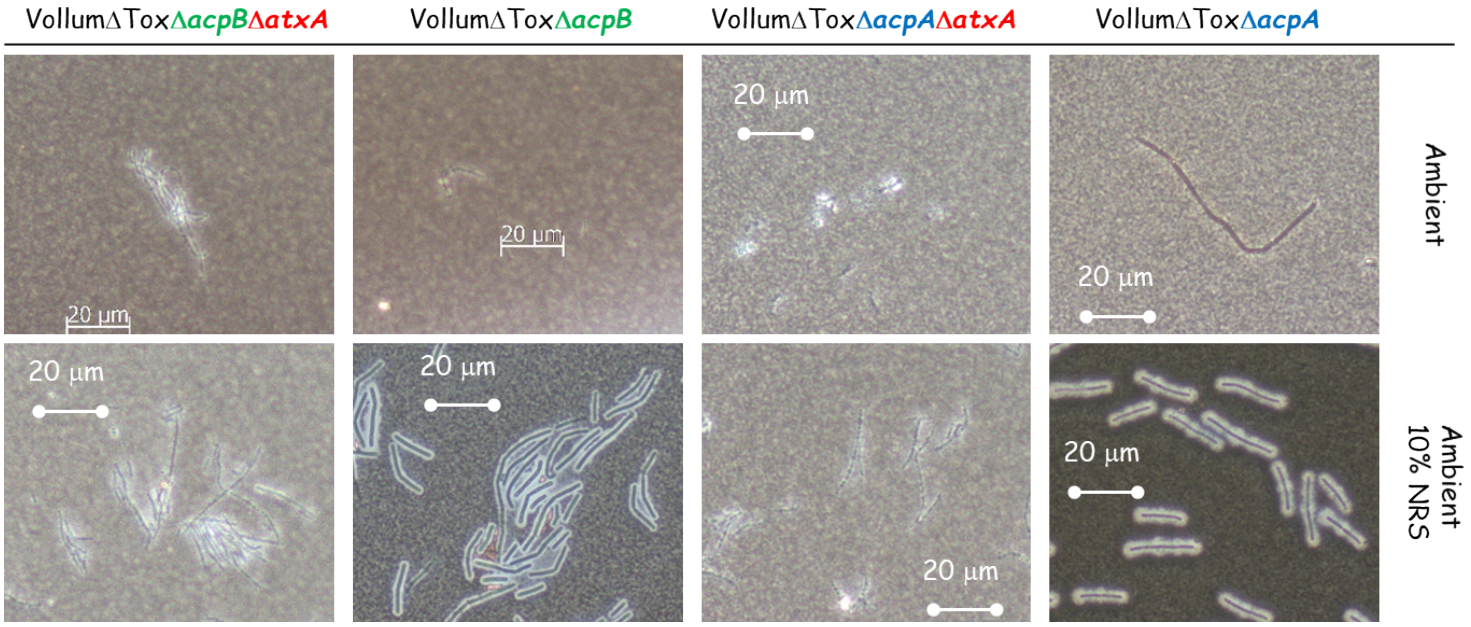


282 Figure 8. Effect of AtxA on Capsule accumulation in response to 10% NRS in ambient atmosphere. Spores
283 of the different mutants (top panel) were seeded into 100µl of DMEM as is or supplemented with 10% NRS and

incubated at 37°C in ambient 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).

Discussion

For successful invasion the pathogen mast regulates 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) of physical (pH or temperature for example). *B. anthracis* naturally infect humans following spore inhalation, contact with broken skin or ingestion of undercook 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]. HCO_3^- /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 addition *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.

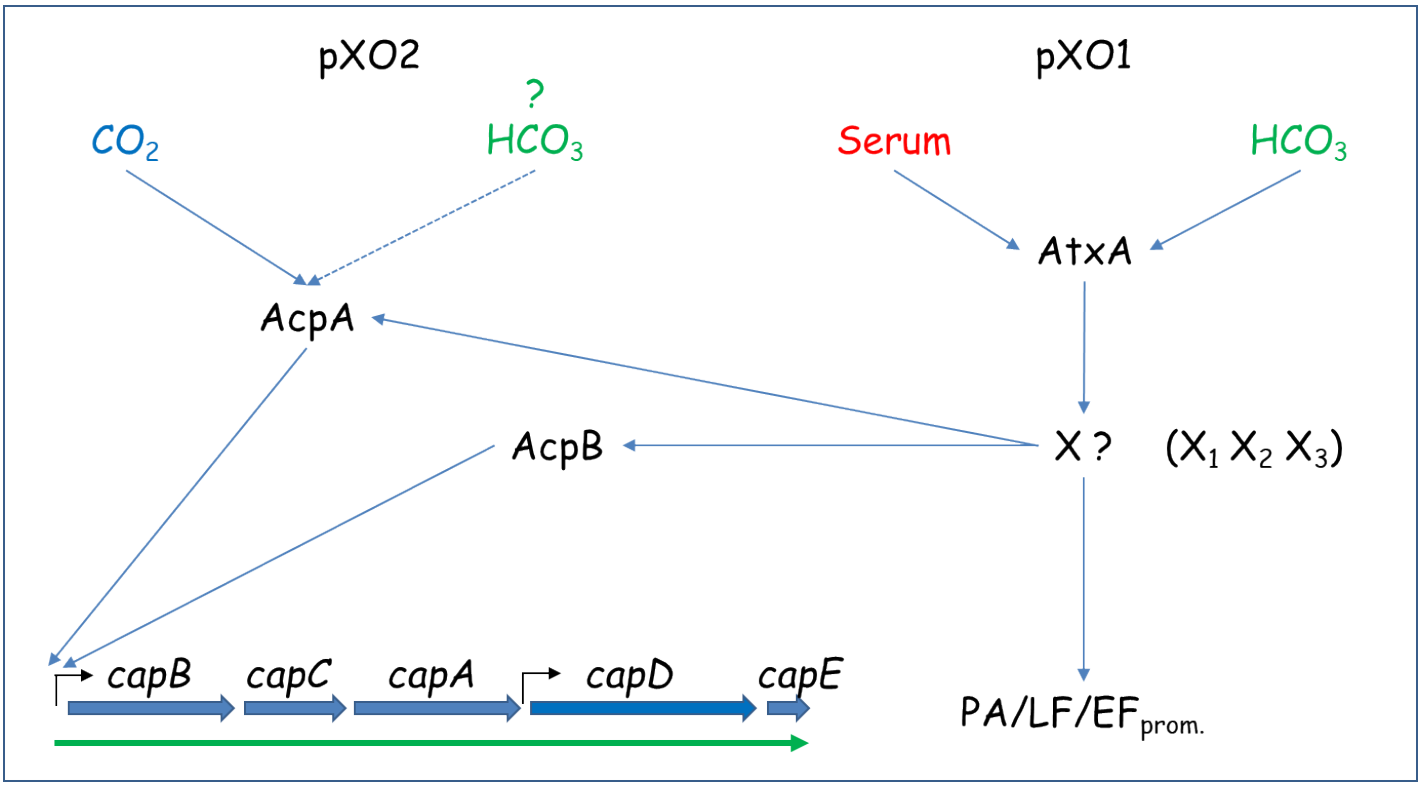
The parameter of soluble CO_2 is influenced by multiple parameters such as surface area to volume ratio and aerobic bacterial growth. Therefore, normal growth conditions were determined as 100μl media/well of a 96 well tissue culture plate for 24h at 37°C in ambient condition (**Figure 1**). This baseline 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 it the mutants that did not express AtxA (VollumΔ*atxA* and VollumΔpXO1). 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**). Toxins 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**) were 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 priestly reported mutant stain which is missing pXO2 and have a chromosomal copy of the CAP operon regulated by a *pag* promotor [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 recreation and capsule accumulation after short incubation of 5h. HCO_3^- was not as robust as NRS in inducing toxins secretion. Examining the PA concertation 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 reviles 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 then HCO_3^- in inducing toxin secretion, two process that are *atxA* dependent. The *atxA* independent 10% CO_2 atmosphere appeared more effective then *atxA* dependent serum for capsule accumulation (**Figure 5**).

Two major regulators; AcpA and AcpB controle capsule biosynthetic 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 promotor. 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 (**Figure7**). AcpA activity is not affected by the absence of *atxA* in the presence of CO_2, (**Figure7**) 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 eliminate 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 form pagolysis. Toxin production in this stage in 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 CO_2 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 CO_2, Serum and HCO_3^- on capsule production and toxin secretion.

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References

1. Dixon TC, Meselson M, Guillemin J, Hanna PC. Anthrax. New England Journal of Medicine. 1999;341(11):815-26. doi: 10.1056/nejm199909093411107. PubMed PMID: 10477781.
2. Hanna P. Anthrax pathogenesis and host response. Curr Top Microbiol Immunol. 1998;225:13-35. Epub 1997/12/05. PubMed PMID: 9386326.
3. Swartz MN. Recognition and management of anthrax--an update. N Engl J Med. 2001;345(22):1621-6. Epub 2001/11/13. doi: 10.1056/NEJMra012892. PubMed PMID: 11704686.
4. Candela T, Fouet A. Bacillus anthracis CapD, belonging to the gamma-glutamyltranspeptidase family, is required for the covalent anchoring of capsule to peptidoglycan. Mol Microbiol. 2005;57(3):717-26. Epub 2005/07/28. doi: 10.1111/j.1365-2958.2005.04718.x. PubMed PMID: 16045616.
5. Candela T, Mock M, Fouet A. CapE, a 47-amino-acid peptide, is necessary for Bacillus anthracis polyglutamate capsule synthesis. J Bacteriol. 2005;187(22):7765-72. Epub 2005/11/04. doi: 10.1128/JB.187.22.7765-7772.2005. PubMed PMID: 16267300; PubMed Central PMCID: PMCPMC1280324.
6. Heine HS, Shadomy SV, Boyer AE, Chuvala L, Riggins R, Kesterson A, et al. Evaluation of Combination Drug Therapy for Treatment of Antibiotic-Resistant Inhalation Anthrax in a Murine Model. Antimicrob Agents Chemother. 2017;61(9). Epub 2017/07/12. doi: 10.1128/AAC.00788-17. PubMed PMID: 28696235; PubMed Central PMCID: PMCPMC5571330.
7. Candela T, Fouet A. Poly-gamma-glutamate in bacteria. Molecular Microbiology. 2006;60(5):1091-8. doi: doi:10.1111/j.1365-2958.2006.05179.x.
8. Wu R, Richter S, Zhang RG, Anderson VJ, Missiakas D, Joachimiak A. Crystal structure of Bacillus anthracis transpeptidase enzyme CapD. J Biol Chem. 2009;284(36):24406-14. Epub 2009/06/19. doi: 10.1074/jbc.M109.019034. PubMed PMID: 19535342; PubMed Central PMCID: PMCPMC2782033.
9. Richter S, Anderson VJ, Garufi G, Lu L, Budzik JM, Joachimiak A, et al. Capsule anchoring in Bacillus anthracis occurs by a transpeptidation reaction that is inhibited by capsidin. Mol Microbiol. 2009;71(2):404-20. Epub 2008/11/20. doi: 10.1111/j.1365-2958.2008.06533.x. PubMed PMID: 19017271; PubMed Central PMCID: PMCPMC2770442.
10. Klimpel KR, Arora N, Leppla SH. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. Mol Microbiol. 1994;13(6):1093-100. Epub 1994/09/01. doi: 10.1111/j.1365-2958.1994.tb00500.x. PubMed PMID: 7854123.
11. Tang W-J, Guo Q. The adenylyl cyclase activity of anthrax edema factor. Molecular Aspects of Medicine. 2009;30(6):423-30.
12. Liu S, Moayeri M, Leppla SH. Anthrax lethal and edema toxins in anthrax pathogenesis. Trends Microbiol. 2014;22(6):317-25. Epub 2014/04/02. doi: 10.1016/j.tim.2014.02.012. PubMed PMID: 24684968; PubMed Central PMCID: PMCPMC4041834.
13. Ebrahimi CM, Kern JW, Sheen TR, Ebrahimi-Fardooee MA, van Sorge NM, Schneewind O, et al. Penetration of the blood-brain barrier by Bacillus anthracis requires the pXO1-encoded BslA protein. J Bacteriol. 2009;191(23):7165-73. Epub 2009/10/13. doi: 10.1128/JB.00903-09. PubMed PMID: 19820089; PubMed Central PMCID: PMCPMC2786561.
14. Uchida I, Hornung JM, Thorne CB, Klimpel KR, Leppla SH. Cloning and characterization of a gene whose product is a trans-activator of anthrax toxin synthesis. J Bacteriol. 1993;175(17):5329-38. Epub 1993/09/01. doi: 10.1128/jb.175.17.5329-5338.1993. PubMed PMID: 8366021; PubMed Central PMCID: PMCPMC206586.
15. Dai Z, Sirard JC, Mock M, Koehler TM. The atxA gene product activates transcription of the anthrax toxin genes and is essential for virulence. Mol Microbiol. 1995;16(6):1171-81. Epub 1995/06/01. doi: 10.1111/j.1365-2958.1995.tb02340.x. PubMed PMID: 8577251.
16. Drysdale M, Bourgogne A, Hilsenbeck SG, Koehler TM. atxA controls Bacillus anthracis capsule synthesis via acpA and a newly discovered regulator, acpB. J Bacteriol. 2004;186(2):307-15. Epub

2004/01/02. doi: 10.1128/jb.186.2.307-315.2004. PubMed PMID: 14702298; PubMed Central PMCID: PMCPMC305762.

17. Sittner A, Bar-David E, Glinert I, Ben-Shmuel A, Schlomovitz J, Levy H, et al. Role of acpA and acpB in Bacillus anthracis capsule accumulation and toxin independent pathogenicity in rabbits. Microb Pathog. 2021;155:104904. Epub 2021/05/01. doi: 10.1016/j.micpath.2021.104904. PubMed PMID: 33930422.

18. Fouet A. AtxA, a Bacillus anthracis global virulence regulator. Res Microbiol. 2010;161(9):735-42. Epub 2010/09/25. doi: 10.1016/j.resmic.2010.09.006. PubMed PMID: 20863885.

19. Levy H, Glinert I, Weiss S, Sittner A, Schlomovitz J, Altboum Z, et al. Toxin-independent virulence of Bacillus anthracis in rabbits. PLoS One. 2014;9(1):e84947. Epub 2014/01/15. doi: 10.1371/journal.pone.0084947. PubMed PMID: 24416317; PubMed Central PMCID: PMCPMC3885664.

20. Fouet A, Mock M. Differential influence of the two Bacillus anthracis plasmids on regulation of virulence gene expression. Infect Immun. 1996;64(12):4928-32. Epub 1996/12/01. doi: 10.1128/IAI.64.12.4928-4932.1996. PubMed PMID: 8945528; PubMed Central PMCID: PMCPMC174470.

21. Bertin M, Chateau A, Fouet A. Full expression of Bacillus anthracis toxin gene in the presence of bicarbonate requires a 2.7-kb-long atxA mRNA that contains a terminator structure. Res Microbiol. 2010;161(4):249-59. Epub 2010/04/03. doi: 10.1016/j.resmic.2010.03.003. PubMed PMID: 20359529.

22. Vietri NJ, Marrero R, Hoover TA, Welkos SL. Identification and characterization of a trans-activator involved in the regulation of encapsulation by Bacillus anthracis. Gene. 1995;152(1):1-9. Epub 1995/01/11. doi: 10.1016/0378-1119(94)00662-c. PubMed PMID: 7828912.

23. Levy H, Weiss S, Altboum Z, Schlomovitz J, Glinert I, Sittner A, et al. Differential Contribution of Bacillus anthracis Toxins to Pathogenicity in Two Animal Models. Infect Immun. 2012;80(8):2623-31. Epub 2012/05/16. doi: IAI.00244-12 [pii]
10.1128/IAI.00244-12. PubMed PMID: 22585968.

24. Levy H, Fisher M, Ariel N, Altboum Z, Kobiler D. Identification of strain specific markers in Bacillus anthracis by random amplification of polymorphic DNA. FEMS Microbiol Lett. 2005;244(1):199-205. PubMed PMID: 15727841.

25. Levy H, Weiss S, Altboum Z, Schlomovitz J, Rothschild N, Glinert I, et al. The effect of deletion of the edema factor on Bacillus anthracis pathogenicity in guinea pigs and rabbits. Microb Pathog. 2012;52(1):55-60. Epub 2011/10/25. doi: S0882-4010(11)00184-7 [pii]
10.1016/j.micpath.2011.10.002. PubMed PMID: 22020310.

26. Welkos SL. Plasmid-associated virulence factors of non-toxigenic (pX01-) Bacillus anthracis. Microb Pathog. 1991;10(3):183-98. Epub 1991/03/01. doi: 10.1016/0882-4010(91)90053-d. PubMed PMID: 1654495.

27. Chiang C, Bongiorni C, Perego M. Glucose-dependent activation of Bacillus anthracis toxin gene expression and virulence requires the carbon catabolite protein CcpA. J Bacteriol. 2011;193(1):52-62. Epub 2010/10/26. doi: 10.1128/JB.01656-09. PubMed PMID: 20971911; PubMed Central PMCID: PMCPMC3019961.

28. McCall RM, Sievers ME, Fattah R, Ghirlando R, Pomerantsev AP, Leppla SH. Bacillus anthracis Virulence Regulator AtxA Binds Specifically to the pagA Promoter Region. J Bacteriol. 2019;201(23). Epub 2019/10/02. doi: 10.1128/JB.00569-19. PubMed PMID: 31570528; PubMed Central PMCID: PMCPMC6832065.

29. Guignot J, Mock M, Fouet A. AtxA activates the transcription of genes harbored by both Bacillus anthracis virulence plasmids. FEMS Microbiol Lett. 1997;147(2):203-7. Epub 1997/02/15. doi: 10.1111/j.1574-6968.1997.tb10242.x. PubMed PMID: 9119194.

30. Drysdale M, Bourgogne A, Koehler TM. Transcriptional analysis of the Bacillus anthracis capsule regulators. J Bacteriol. 2005;187(15):5108-14. Epub 2005/07/21. doi: 10.1128/JB.187.15.5108-5114.2005. PubMed PMID: 16030203; PubMed Central PMCID: PMCPMC1196023.