nature portfolio

Peer Review File

Helical ultrastructure of the L-ENA spore aggregation factor of a Bacillus paranthracis foodborne outbreak strain

Editorial Note: Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, the authors utilized cryoID, alphafold modelling and molecular genetic approaches to characterize the composition, genetic arrangement and expression profile, and protein structure of the L-ENA spore appendages of the of the food-poisoning outbreak strain Bacillus paranthracis NVH 0075-95. The manuscript is well written, the results are logically presented and well supported by the Figures (both in the main text and the supplemental material). My comments and concerns are relatively minor.

Specific comments:

1. Line 56: I am not familiar with publications identifying spore appendages of Bacillus anthracis. Please provide a reference citation for this.

2. Line 61: diphtheriae (typo present)

3. Figure 4c and lines 233-234: Under these culture conditions, when do the cells display the asymmetric septation event indicative of early sporulation events and when do spores first become visible in the mother cells? This is important since no vegetative gene or sporulation gene controls are indicated in the Figure.

4. Figure 5: With the delta ena3A mutant, how can you have no appendages but still have ruffles?

5. Supporting figure 9d: The B. anthracis N-terminal cleavage site is not correct. It was reported to be after amino acid 19, not 20.

6. Lines 308-313: Actually the sequences responsible for exosporium targeting of BclA was first reported in 2008 (doi: 10.1111/j.1365-2958.2008.06420.x). BclA is proteolytically processed to remove the first 19 amino acids, not 20 (line 310). To date, there is no definitive published data indicating that the cleavage of BclA results in attachment to BxpB.

7. For the supplemental material Tables, provide more complete reference citations.

Reviewer #2 (Remarks to the Author):

What are the noteworthy results?
o The authors have described the L-ENA structures found on the Bacillus paranthracis NVH 0075-95 spore in detail and have identified three genes encoding the components of L-ENA and characterised the role of each gene in the formation of the L-ENA structures on the exosporium. They have also shown that these three genes are expressed under conditions known to promote sporulation. As well as Bacillus paranthracis NVH 0075-95 the authors have identified 62 or B. cereus sensu lato strains carrying single or multiple copies of the ena3 gene cluster.
Will the work be of significance to the field and related fields? How does it compare to the established literature?
o This work could be significant to the identification and characterization of endospore appendages in other Bacillus as wells as other sporulating bacteria. This work could also be significant to studies focusing on understanding the interaction of spores with their environment as well as the role spores play during infection.
Does the work support the conclusions and claims, or is additional evidence needed?
o I don’t think the title is backed up by the results; the authors have not demonstrated that the L-ENA

structures they have described are virulence factors. In the discussion the authors state: 'Given that the ena3a gene cluster is predominantly found in pathogenic Bacilli we anticipate that L-ENA fibers could be considered as a novel class of secondary effect virulence factors through their impact on spore aggregation, and by extension biofilm formation.' I don't feel that this observation is enough to back up the statement of the title that these are 'virulence factors'. I agree that it is likely that, if present in a biofilm, they could contribute to structural integrity and therefore influence virulence, but this is just a hypothesis and not supported by their results. Since the authors have mutant strains of the three ena3 genes, could the biofilm properties or at least the aggregation ability of these mutants be tested?
o All other claims made by the authors were supported by the results.
Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?
o I assume that the results to support the conclusion that 'the ena3a gene cluster is predominantly found in pathogenic Bacilli' are presented in paragraph beginning line 201? To aid interpretation of these results, it would be useful to have more information about the 62 strains such as accession numbers and any connected meta data given in the supplementary. Are you making the assumption that these 62 strains are pathogenic based on the species they belong to or is their further information that you have about them? It is my understanding that not all B. cereus strains are necessarily pathogenic.
o The results presented in lines 239-242 could be supported with microscopy images showing that under the growth conditions used that cells are still vegetative at in the first 8 hours and sporulate at 12 hours.
Is the methodology sound? Does the work meet the expected standards in your field?
o Yes
Is there enough detail provided in the methods for the work to be reproduced?
o Yes
Minor edits and comments:
o Line 209: the B. cereus s. l. abbreviation has not been defined in the above text. It is also written in full in the later text. Please correct this.
o Line 220: where you say 'many of the isolates' could you give a specific number rather than 'many'.
o Lines 242-244: It's interesting that ena3a had higher expression than l-bclA when they are expressed bicistronically, I would expect it would be the other way around as l-blcA is upstream of ena3a. Do you also find this unexpected?

Reviewer #3 (Remarks to the Author):

This paper describes the structure of a type of fibrous appendage (called L-ENA, or "ladder-like endospore appendages") associated with the exosporium in Bacillus paranthracis spores. The L-ENA fibers are related to a previously characterized fiber called S-ENA or "staggered ENA".

Using cryo-EM structure determination, they were able to identify the nature of the protein subunits of the L-ENA. While the limited resolution (5.8Å) precluded de novo model building, they were able to identify the protein (Ena3A) by comparison with the ENA1A/B subunits of the S-ENA. This was confirmed by overexpression of Ena3 in E. coli, leading to the formation of fiber-like structures resembling L-ENA. These fibers were reconstruction to a resolution of 3.3Å. The fibers were remarkably stable at high temperature, or in the presence of denaturants like urea or SDS.

Genomic analysis indicated that the gene encoding Ena3A is part of a cluster containing genes exsL and l-bclA that are found in a mobile genetic element. ExsL anchors the L-ENA fiber to the exosporium while L-BclA is a BclA-like protein that forms a "ruffle" at the tip of the fiber.

This is an interesting study and a useful contribution to our understanding of endospores. The

manuscript is well written and clearly presented and nicely illustrated. The cryo-EM is executed carefully and supports the conclusions. I have only a few comments:

The introduction is a bit unclear (lines 67-85) as to whether the S-ENA were the subject of this study or previously characterized. This only becomes clear in the first part of the results section (line 117), where they specify that they used Ena1B as a template for modeling of the L-ENA fibers. Clearly, they already had some idea that the S-ENA and L-ENA fibers might be structurally related. This should be clarified in the introduction and the results section.

The authors mention the characterization of BclA by the Turnbough group, as well as the structural study of the exosporium by Kailas et al (2011). It may be worth noting that the Turnbough lab has published a cryo-EM study of the B. anthracis exosporium (Rodenburg et al 2014) and also studied other aspects of the exosporium that may be relevant to this study, including Tan et al 2011 and Chattopadhyay et al 2023.

Could the authors comment further on the role of the exosporium in pathogenicity? B. anthracis spores that lack the exosporium are equally infectious as the wildtype spores in a mouse model. How can this be reconciled with the fact that the exosporium is conserved in most pathogenic bacilli and presumably plays a role under certain conditions?

We appreciate the constructive and positive feedback that we received from all three reviewers. We address all of the reviewers' comments point by point below:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In this study, the authors utilized cryoID, alphafold modelling and molecular genetic approaches to characterize the composition, genetic arrangement and expression profile, and protein structure of the L-ENA spore appendages of the of the food-poisoning outbreak strain Bacillus paranthracis NVH 0075-95. The manuscript is well written, the results are logically presented and well supported by the Figures (both in the main text and the supplemental material). My comments and concerns are relatively minor.

Specific comments:

1. Line 56: I am not familiar with publications identifying spore appendages of Bacillus anthracis. Please provide a reference citation for this.

- ⇨ 10.15252/embj.2020106887: using bioinformatics we showed that the S-ENA gene cluster is also found in *Bacillus anthracis* stains. We also performed negative stain imaging on [Redacted] (unpublished data, see figure below).

[Redacted]

[Redacted]

2. Line 61: diphtheriae (typo present)

- ⇨ Corrected

3. Figure 4c and lines 233-234: Under these culture conditions, when do the cells display the asymmetric septation event indicative of early sporulation events and when do spores first become visible in the mother cells? This is important since no vegetative gene or sporulation gene controls are indicated in the Figure.

- ⇨ This is a valid remark. We performed time-course fluorescence microscopy imaging to track the sporulation process as a function of time. Under the culturing conditions we employed, there was no asymmetric septation within the first five hours of cultivation. Asymmetric septation began to appear at the 6th hour, and by 8th hour, nearly all the cells had formed asymmetric septa (see updated Supporting Figure 7). By the 9th hour, the processes of asymmetric division and engulfment had reached completion. From the 10th hour onward, the appearance of phase-bright bodies became increasingly prominent, and by the 12th hour, the cell population was predominantly composed of cells containing phase-bright bodies. This trend continued until the 14th hour, during which the majority of cells exhibited very bright bodies, and spore maturation continued, with matured spores beginning to be released by the16th hour.
  - ➔ This data was added to the manuscript under section "Distribution and expression of the *ena3A* gene cluster".

4. Figure 5: With the delta ena3A mutant, how can you have no appendages but still have ruffles?

- ⇨ That is indeed impossible and reflects a typo on our part. The table has been updated.

5. Supporting figure 9d: The B. anthracis N-terminal cleavage site is not correct. It was reported to be after amino acid 19, not 20.

- ⇨ This is correct. We apologize for the oversight and have corrected the figure.

6. Lines 308-313: Actually the sequences responsible for exosporium targeting of BclA was first reported in 2008 (doi: 10.1111/j.1365-2958.2008.06420.x). BclA is proteolytically processed to remove the first 19 amino acids, not 20 (line 310). To date, there is no definitive published data indicating that the cleavage of BclA results in attachment to BxpB.

- ⇨ We have altered the residue numbering and replaced the corresponding reference with doi: 10.1111/j.1365-2958.2008.06420.x

7. For the supplemental material Tables, provide more complete reference citations.

- ⇨ Reference citations have been added to the supplemental material

Reviewer #2 (Remarks to the Author):

What are the noteworthy results?

o The authors have described the L-ENA structures found on the Bacillus paranthracis NVH 0075-95 spore in detail and have identified three genes encoding the components of L-ENA and characterised the role of each gene in the formation of the L-ENA structures on the exosporium. They have also shown that these three genes are expressed under conditions known to promote sporulation. As well as Bacillus paranthracis NVH 0075-95 the authors have identified 62 or B. cereus sensu lato strains carrying single or multiple copies of the ena3 gene cluster.
Will the work be of significance to the field and related fields? How does it compare to the established literature?

This work could be significant to the identification and characterization of endospore appendages in other *Bacillus* as wells as other sporulating bacteria. This work could also be significant to studies focusing on understanding the interaction of spores with their environment as well as the role spores play during infection.

Does the work support the conclusions and claims, or is additional evidence needed?

o I don't think the title is backed up by the results; the authors have not demonstrated that the L-ENA structures they have described are virulence factors. In the discussion the authors state: 'Given that the ena3a gene cluster is predominantly found in pathogenic Bacilli we anticipate that L-ENA fibers could be considered as a novel class of secondary effect virulence factors through their impact on spore aggregation, and by extension biofilm formation.' I don't feel that this observation is enough to back up the statement of the title that these are 'virulence factors'. I agree that it is likely that, if present in a biofilm, they could contribute to structural integrity and therefore influence virulence, but this is just a hypothesis and not supported by their results. Since the authors have mutant strains of the three ena3 genes, could the biofilm properties or at least the aggregation ability of these mutants be tested?

- ⇨ Testing the aggregation ability of the mutant strains is an excellent suggestion. Leading up to the submission of the current manuscript, we were working on finalizing another manuscript that focuses on role of L-ENA and L-BclA in the aggregation behaviour of *Bacillus paranthracis*. That manuscript has since been submitted to biorxiv (https://www.biorxiv.org/content/10.1101/2024.04.22.590507v1) and provides clear evidence that spore-spore coupling and aggregation is driven by the display of L-BclA – be it on the termini of L- or S-ENA. We did not test the effect of spore-spore aggregation on virulence, so we agree with the reviewers assessment that claims or statements regarding L-ENA's contribution to virulence remain premature. To that end, we have removed all strong statements related to spore virulence and limit the discussion to the involvement of L-ENA on spore-spore aggregation, with the suggestion that one could reasonably expect secondary effects (e.g. stronger biofilm -> more persistence / resilience) but that such hypotheses require future research.

⇨ We also propose to change the title: “CryoEM structure of a novel class of spore aggregation factors on the foodborne outbreak strain *Bacillus paranthracis* NVH 0075-95”

o All other claims made by the authors were supported by the results. Are there any flaws in the data analysis, interpretation and conclusions? Do these prohibit publication or require revision?

I assume that the results to support the conclusion that ‘the ena3a gene cluster is predominantly found in pathogenic Bacilli’ are presented in paragraph beginning line 201? To aid interpretation of these results, it would be useful to have more information about the 62 strains such as accession numbers and any connected meta data given in the supplementary. Are you making the assumption that these 62 strains are pathogenic based on the species they belong to or is their further information that you have about them? It is my understanding that not all B. cereus strains are necessarily pathogenic.

⇨ The accession numbers and any associated metadata is accessible at https://microreact.org/project/uzm4JFrrsCPZeRnMpRqvvf-supplementary-figure-9-ena3-paper. The link was already provided in the caption of Supporting Figure 6 but we now also refer to it in the main text. Indeed, not all strains in the *B.c.* group are pathogenic. Our statements regarding the pathogenicity of the ena3 carrying strains stems from species information, i.e. some strains within the *B. cereus* s.l. group can cause disease in humans, animals, and insects. However, since we do not have detailed information regarding the pathogenicity of the ena3 carrying strains we have removed the term from the abstract, the sentence now reads: “The role of ENA fibers in spore-spore interaction and the distribution of L-ENA operon as mobile genetic elements in *B. cereus* s.l. strains suggest that L-ENA fibers may increase the survival, spread and virulence of these strains.”

o The results presented in lines 239-242 could be supported with microscopy images showing that under the growth conditions used that cells are still vegetative at in the first 8 hours and sporulate at 12 hours.

⇨ Indeed. Reviewer 1 raised a similar point regarding the timing of sporulation. We have now also performed time-course fluorescence microscopy analysis of the sporulation process. That data has been made available in Supporting Figure 7, and the results are discussed in section “Distribution and expression of the *ena3A* gene cluster”.

Is the methodology sound? Does the work meet the expected standards in your field?
o Yes

Is there enough detail provided in the methods for the work to be reproduced?
o Yes

Minor edits and comments:

o Line 209: the B. cereus s. l. abbreviation has not been defined in the above text. It is also written in full in the later text. Please correct this.

- ⇨ Thank you for pointing this out, we have corrected this mistake.

o Line 220: where you say ‘many of the isolates’ could you give a specific number rather than ‘many’.

- ⇨ Certainly, we now state “six out of the 14 human clinical isolates carrying *ena3* were isolated from bloodborne infections.”

o Lines 242-244: It’s interesting that ena3a had higher expression than l-bclA when they are expressed bicistronically, I would expect it would be the other way around as l-blcA is upstream of ena3a. Do you also find this unexpected?

- ⇨ At first instance, we were indeed puzzled by this result given the order of the genes and we therefore repeated the experiment multiple times with different primer sets to make sure that the result is robust. Gene expression modulation through mRNA degradation tends to be targeted at the 3’ end, which would lead to higher expression levels for the upstream gene (i.e. l-bclA). We hypothesize that this may not be the case for the *l-bcla*/*ena3a* cistron. Indeed, looking at the work of the Ole Andreas Økstad's group (https://genomebiology.biomedcentral.com/articles/10.1186/gb-2012-13-4-r30), they showed that in *Bacillus cereus*, an equivalent number of genes decay from both the 3’ and 5’ sides. Additionally, they observed that within polycistronic operons, certain open reading frames decay at varying rates, highlighting the crucial role of RNA processing in gene regulation.
- ⇨ We have included this explanation in the manuscript under section “Distribution and expression of the ena3A gene cluster”.

Reviewer #3 (Remarks to the Author):

This paper describes the structure of a type of fibrous appendage (called L-ENA, or “ladder-like endospore appendages”) associated with the exosporium in Bacillus paranthracis spores. The L-ENA fibers are related to a previously characterized fiber called S-ENA or “staggered ENA”.

Using cryo-EM structure determination, they were able to identify the nature of the protein subunits of the L-ENA. While the limited resolution (5.8Å) precluded de novo model building, they were able to identify the protein (Ena3A) by comparison with the ENA1A/B subunits of the S-ENA. This was confirmed by overexpression of Ena3 in E. coli, leading to the formation of fiber-like structures resembling L-ENA. These fibers were reconstruction to a resolution of 3.3Å. The fibers were remarkably stable at high temperature, or in the presence of denaturants like urea or SDS.

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- ⇨ Indeed, we did have some ‘intuition’ that the major subunits of S-ENA and L-ENA would likely belong to the same protein family, but -to be clear- before the start of this project there was no experimental proof to support that hypothesis. We have worked on other Bacillus species and have shown (unpublished) that they can carry multiple different ENA fibers that share no structural or sequence homology. We therefore chose to write the introduction in such a way that we would not inject any personal bias.

The authors mention the characterization of BclA by the Turnbough group, as well as the structural study of the exosporium by Kailas et al (2011). It may be worth noting that the Turnbough lab has published a cryo-EM study of the B. anthracis exosporium (Rodenburg et al 2014) and also studied other aspects of the exosporium that may be relevant to this study, including Tan et al 2011 and Chattopadhyay et al 2023.

- ⇨ We appreciate these helpful additions to our reference list.

Could the authors comment further on the role of the exosporium in pathogenicity? B. anthracis spores that lack the exosporium are equally infectious as the wildtype spores in a mouse model. How can this be reconciled with the fact that the exosporium is conserved in most pathogenic bacilli and presumably plays a role under certain conditions?

- ⇨ It has been assumed that the exosporium plays a role in *B. anthracis* infections as it is the outermost structure of the spores and would make initial contact with host and immune cells. The exosporium is densely covered with the highly glycosylated collagen-like protein BclA. Recently, Norris et al., suggested that using high infectious doses in studies may mask the virulence effects of a *bclA* knockout, which could be detected using a more sensitive LD_{50} test. BclA was found to be dispensable for achieving full virulence in high dosage Sterne (10.1046/j.1365-2958.2000.03000.x) or Ames (10.1128/IAI.01202-06) mice challenge trials. Notably, in another study, a Δ*bclA* Sterne mutant exhibited a 50–70% decrease in LD50 compared to the wild-type Sterne. Moreover, the presence of the exosporium is conserved among pathogenic Bacilli, but its composition differs. For example, BclA of *B. anthracis* is densely glycosylated with a pentasaccharide with anthrose in its distal end. The pentasaccharide with anthrose is exclusively found on the surface of *B. anthracis* spores and not produced by any other *Bacillus* spp. Other *Bacillus* spp. spores, including those of *Bacillus cereus*, has cereose at the terminus of the corresponding

glycan chains. This makes it likely that the exosporium also play specific roles in individual species.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have adequately responded to the concerns and comments raised in the initial review. The revised manuscript describes an important contribution to the field of Bacillus spore biology.

Reviewer #2 (Remarks to the Author):

I am happy that the authors have adequately addressed my concerns.

Reviewer #3 (Remarks to the Author):

It appears that the authors have adequately addressed the concerns by all three reviewers. I have no further issues or concerns about the manuscript.