THE EMBO JOURNAL

Endospore Appendages: A novel pilus superfamily from the endospores of pathogenic Bacilli

Han Remaut, Brajabandhu Pradhan, Mike Sleutel, Janine Liedtke, Toril Lindback, Kristin O'Sullivan, Ann-Katrin Llarena, Ola Brynildsrud, Marina Aspholm, and Ephrem Zegeye
DOI: 10.15252/embj.2020106887

Corresponding authors: Han Remaut (han.remaut@vub.be) , Marina Aspholm (marina.aspholm@nmbu.no)

Review Timeline:

Submission Date:	24th Sep 20
Editorial Decision:	16th Oct 20
Revision Received:	16th Feb 21
Editorial Decision:	11th Mar 21
Revision Received:	29th Mar 21
Accepted:	16th Apr 21

Editor: Ieva Gailite

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you for submitting your manuscript for consideration by The EMBO Journal. We have now received three referee reports on your manuscript, which are included below for your information.

As you will see from the comments, all reviewers appreciate the work and the quality of the data, while also indicating a number of issues that would have to be addressed and clarified in the revised manuscript. Based on these positive evaluations, I would like to invite you to submit a revised version of your manuscript in response to reviewers' comments.

We have extended our 'scooping protection policy' beyond the usual 3 month revision timeline to cover the period required for a full revision to address the essential experimental issues. This means that competing manuscripts published during revision period will not negatively impact on our assessment of the conceptual advance presented by your study. Please contact me if you see a paper with related content published elsewhere to discuss the appropriate course of action.

Please feel free to contact me if you have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to receiving the revised manuscript.

Referee #1:

The manuscript entitled "A novel pilus superfamily from the endospores of pathogenic Bacilli" by Pradhan et al. reports the functional and structural characterisation of a new type of pilus found on Bacillus endospores. This is a very nice work overall, reporting a significant finding (it is not every day that a new pilus is identified), but the text/presentation could be improved, which should not be too difficult.

Major comments

1. The legends to the figures are way too short and imprecise. This needs to be addressed throughout the text.

2. Make it clear throughout the text, including the abstract, that only one ENA has been structurally characterised in this paper.

3. The description of the identification of the subunit is confusing. Only one candidate matches the FCMCTIRY sequence, it is KMP91698.1. The other two proteins, KMP91697.1 and KMP91699.1, are identified because they show sequence homology to this protein. Making this clear would make that portion easier to follow.

4. Fig. S2 should absolutely be in the main text since this is one of the most important points in the paper. Also, the description of the gel in panel C could be clearer, with the processed and unprocessed bands indicated by arrows. Since there is no difference +/- β-mercaptoethanol, that lane could be removed to facilitate understanding. Moreover, unlike what is stated, the 2 kDa could not correspond to the 6xHis tag only, which has MW of 840 Da. Is this perhaps tag + TEV cutting

site?

5. An important point that is not addressed in the paper is what is the structure of the ruffles? I understand this is beyond the scope of this paper, but this should be explicit.

6. The only experiment that is missing in the paper is an important control for Fig S3E. There is no evidence that the antibodies are specific for the different subunits, which should be provided. Otherwise the conclusions drawn from the immunogold data could be wrong. These experiments could also test antibody sensitivity, which would help the authors figuring out whether the absence of Ena1C is due to the low abundance of this protein, or simply due to a sub-par antibody. This could and should be discussed in that part of the text. Also, the immunogold data do not show, as stated on lines 195-196, that "native S-type Enas show a mixed Ena1A and Ena1B composition". They show that both 1A and 1B are subunits, but they do not say that they are in the same pili. It is possible that two different homo-polymeric S-types exist, composed of exclusively of one subunit or the other.

7. Fig 5. The length results in panel B, for L- and S-type should be separated. Combining them makes this figure confusing because of the vastly different scales for the two types of filaments.

8. The section describing the phylogenetic analysis is unnecessarily long and should be shortened. Also, it could be simplified for the non-experts. For example, the rational link between paragraphs 1 and 2 is unclear at best.

Other comments

1. line 21: the first time the Enas acronym is introduced underline the corresponding letter in "endospore appendages" to make it obvious what the acronym means. Also, because there are at least two different Enas (S- and L-types) and this papers report the identification and structural characterisation of only one of them, it is necessary to slightly modify the summary to make this point clear. Perhaps call the two Enas ENA-S and ENA-L, and mention that just ENA-S has been characterised in this paper?

2. line 25: β-augmentation is not a common concept. It would be good to explain ina few words what this actually means.

3. line 29: "different eco- and pathotypes" of what? This needs to be spelled out.

4. line 35: "bacteria belonging to the phylum Firmicutes" is too general. Not every Firmicutes produces endospores, far from it actually. Also, Firmicutes should not be in italics.

5. lines 50-51: "with multiple micrometers long and a few nanometers wide filamentous appendages" is confusing, as it seems to suggest that there are two types of filaments. Many µm-long filaments, and less nm-wide ones, which is not what the authors intend. Dropping "few" from the above sentence would avoid any confusion.

6. lines 71-72: The sentence "The genetic identity of the S-type Enas was deduced from the structural model" is difficult to understand. Replace by "The identity of the subunit of S-type Ena was deduced from the structural model".

7. Fig. 1. The order of the panels in this Fig does not reflect the order in which they are mentioned in

the text. On line 92, there is mention of "scales pointing down". This is not obvious from this Fig. Moreover, in Fig. 2 the scales seem to be pointing up. The terminal extensions or ruffles ins ENA-S are difficult to see in Fig. 1C. Could the contrast be increased a bit to help visualisation? It should be clearly spelled out in the legend what the distances in Fig. 1C and Fig. 1D insets are. Giving names to the two ENA (see point 1) is particularly important in this part of the text because the results are mainly for ENA-S (e.g. Fig. S1), which is not clearly stated. Also, in the legend to Fig. 1E, its says n=1,023, but there are far more individual data points for the length measurements than for the number of filaments/endospore. Why?

8. line 134: considering the resolution of the structure, the use of 5 decimals "3.22937 Å and 31.0338" is not necessary. One decimal seems more than enough. Same applies to numbers on line 172.

9. line 145: delete "locus" at the end of this sentence.

10. Legend to Fig. 2 is mis-labelled: it is panel E, not F.

11. line 259. "mutually" is not right the word, replace by "all".

12. line 322: what is the evidence that it has been "misclassified"?

13. lines 427-428: "electron transfer" should be deleted as it is now known that type IV pili of Geobacter are not involved (doi: 10.1016/j.cell.2019.03.029).

14. legend to Fig. 3: there are more than 19 helical turns in orange on that Fig. unlike what is stated in the legend.

15. legend to Fig. 4: shouldn't it be "RT-PCR" instead of "PCR" on line 512? Line 517, the line is not "dotted", unlike stated here. Line 518, these are not "whiskers" but simple error bars.

16. line 551: mention what "Nycodenz" is.

17. line 562: "kept" should be replaced by "incubated".

18. line 696: "formvr" is a typo.

19. Supplemental information, line 49: correct typo in "loose".

20. Supplemental information, line 58: evidence should be provided that the staining is "specific". See point 6 in Major comments.

Referee #2:

This manuscript reports the discovery and structural characterization of a new family of pili that are found on endospores of a large number of Bacillus species and a few other species outside of the Bacillus genus. Termed Enas, for endospore appendages, the authors identified two structurally and compositionally distinct pili, the "staggered-type" (S-type) and "Ladder-like" (L-type). They focus mainly on the S-type pili, solving the structure by CryoEM at a resolution sufficient to identify a short polypeptide that enabled identification of the genetic locus encoding S-type pili. Three

proteins, Ena1A, Ena1B, and Ena1C, are the building blocks of S-type pili, where Ena1A and Ena1B appear to randomly assemble into the pilus; how Ena1C contributes to pilus assembly is not yet defined. Both S-type and L-type pili are completely distinct from other pili known to be produced by Gram-positive bacteria, which include sortase-dependent pili and type IV pili that are produced by vegetative cells. Notably, the pilin subunits are tethered together via disulfide crosslinks via a strand donation mechanism reminiscent of that described for Pap/Type I pili of Gram-negative bacteria except that the latter are not disulfide crosslinked. Strikingly purified Ena1A subunits naturally assemble as pili whose structures are highly similar to the S-type pili found on endospores. Overall, this work is well developed and describes structural, compositional, and morphogenetic details of a novel family of pili associated with endospores of Gram-positive bacteria. In fact, these pili were visualized previously but all efforts till now failed in detailed characterization. Thus, the work significantly advances our knowledge of a group of phylogenetically widely distributed pili that likely specifically associate with endospores. The manuscript is very well written and is readily accessible to a broad readership. I have only a few minor comments for the authors to address:

1. Pg. 2, L. 55. Spell out TEM at first use.

2. Fig. 4. It's somewhat surprising that deletions of dedA and 1232 were not analyzed given the proximity of these genes to the ena genes and results of the qRT-PCR? It's a simple extension of the current studies to evaluate whether the encoded proteins also contribute to pilus assembly, especially since the composition of tip fibrils remains unknown.

3. Fig. 2 legend. Reorganize the (A,B) and then (A) and (B) portions of the first sentence. (F) should be (E).

4. It would be a nice complement to the PCR data to see levels of 1A, B, and C proteins at the different time points by western blotting. This would validate that transcription correlates with protein synthesis, which is often not the case.

5. Pg. 9, L. 260 & Fig. 5. Why was the Deltaena1B mutant complemented with ena1A/1B expression, this results in overproduction of both 1A and 1B. What is the phenotype of transexpressing just 1B? Also, although likely, there is no evidence that transexpression yields overproduction of Ena1A and Ena1B. This should be validated by westerns.

6. In my version of the manuscript, there is no Fig. S5 that corresponds to that referred to in the text, pg. 9. Are Fig. 5 and S5 the same? If so, lettering of the images needs to be revised.

7. The question of whether the impact on exosporium production and S-type Ena entrapment accompanying Ena1A/1B expression relates to timing or abundance of expression could easily be answered by monitoring phenotypic consequences of gene induction at different times in the growth curve.

8. Supp. Pg. 1. L. 17. Change 'loose' to 'lose'.

9. Suppl. Fig. S2. It's interesting that TEV or b-ME treatment have the same effect on Ena1B migration. Explanation?

10. Discussion, Pg. 13, L. 374. There are, however, Gram-positive pili in which the pilin subunits have intramolecular disulfide bonds. This should be mentioned/referenced.

11. Discussion, Pg. 14. L. 407. Can pilus production on vegetative cells be aberrantly stimulated by induction of ena1A/B genes from an inducible promoter? If so, this would allow for the possibility but indicate that pilus production on endospores is controlled at the transcriptional level presumably by signals that remain to be identified.

12. The discussion is a bit redundant with sections of the Results and could be shortened.

Referee #3:

The manuscript by Pradhan and colleagues describes the structural and molecular characterization of a novel fiber on the surface of spores generated by Gram-positive organisms. The work is beautifully and carefully done. Some sections of the manuscript are somewhat dense and a bit too descriptive, but apart from that, it was a pleasurable read. There are some glitches, including the absence of what looks like an important figure, as well as the lack of comparisons with other well-studied Gram-positive pili, but in general this manuscript presents data regarding a poorly described bacterial surface appendage, and provides a clear addition to knowledge on such structures. Some more specific comments/questions can be found below.

Readers may not be familiar with the molecular organization of endospores, and a schematic figure that aids in the comprehension of lines 41-50 would also be helpful for the understanding of Fig. 1a.

Are Enas-encoding genes constitutively expressed, generating spores that are always covered in fibers, or is there an expression activation mechanism?

Lines 91-92: '... S-type Enas ... give a polar, staggered appearance ... with alternating scales pointing down to the spore surface'. Could this be highlighted directly in the figure to facilitate comprehension?

Lines 102-103: in addition to characterizing fibers biochemically, It could be of interest for authors to characterize the fibers by AFM in order to provide a numerical value regarding strength limitations.

Lines 138-140: the experiments described in these lines are absolutely remarkable. Authors deduced the sequence of a peptide from a 3.2 Å EM map and bootstrapped their way to the identification of the building block of the fiber itself, and subsequently, characterization of the fiber. This incredible detail could be highlighted at the end of the introduction or in the discussion.

Lines 168-169: authors should clarify on the images what the mean by 'the distal ruffles seen in ex vivo fibers'

Lines 183-185: the only somewhat worrisome aspect of this experiment is that there is no negative control for labeling done with recEna1A or recEna1b sera. It would be of interest to show that their samples are not simply 'sticky', lighting up other unrelated fibers. Is it possible to do this control experiment?

Line 264: Where is figure S5D?
Line 267: the link to this figure does not seem to be correct, and thus the phrases starting from this line and going on towards the end of the paragraph are very difficult to understand.

Lines 276-344: this section could benefit from shortening & rewriting.

Lines 423-451: authors comment on the continuing lack of knowledge on the function of the Ena fibers. Since they have constructed individual Ena knockout strains, would it be possible to do experiments to verify surface adhesion/structure robustness/binding?

The Ena fibers described in this work are very elegantly described, and authors have gone to great lengths to characterize these poorly known structures. However, there is an 'uncanny' lack of mention of other pilus systems that have been characterized in detail in Gram-positive bacteria and that show some similarities to the work described here. For example, despite the fact that sortase-mediated pilus assembly was cited, pili from the Gram-positive pathogen Streptococcus pneumoniae, that also display covalent bond formation within subunits, individual domains with beta-sandwich folds, and head-to-tail arrangements with recognition of isolated peptides from adjacent domains, as also shown for the Ena fibers, were not mentioned at all and should be discussed. Many interesting papers describe details of these pili (Hilleringmann EMBO J 2009; Izore Structure 2010; Shaik JBC 2015; Gentile JBC 2011). In addition, some space in the discussion should also be given to type IV pili, that were only superficially mentioned (Berry JBC 2019). This does not diminish the novelty of the work presented in the manuscript, and at the same time highlights details of the plethora of interesting fibers present on the surface of Gram-positive organisms.

Minor remark:

Fig. S2b: it is not clear to this reviewer what the blue boxes are supposed to be highlighting or regrouping. Residues such as Leu and Asp are grouped together, as are Thr, Gly and Asn. Authors should revise the input file of the program employed to generate the figure.

Point-by-point author response:

Referee #1:

The manuscript entitled "A novel pilus superfamily from the endospores of pathogenic Bacilli" by Pradhan et al. reports the functional and structural characterisation of a new type of pilus found on Bacillus endospores. This is a very nice work overall, reporting a significant finding (it is not every day that a new pilus is identified), but the text/presentation could be improved, which should not be too difficult.

Major comments

1. The legends to the figures are way too short and imprecise. This needs to be addressed throughout the text.

Author response:
We have expanded the figure legends throughout the manuscript to provide additional clarity.

2. Make it clear throughout the text, including the abstract, that only one ENA has been structurally characterised in this paper.

Author response:
We have highlighted in the abstract and throughout the text that it is the S-type Ena that has been structurally characterized.

3. The description of the identification of the subunit is confusing. Only one candidate matches the FCMCTIRY sequence, it is KMP91698.1. The other two proteins, KMP91697.1 and KMP91699.1, are identified because they show sequence homology to this protein. Making this clear would make that portion easier to follow.

Author response:
This has been changed as requested by the reviewer.

4. Fig. S2 should absolutely be in the main text since this is one of the most important points in the paper. Also, the description of the gel in panel C could be clearer, with the processed and unprocessed bands indicated by arrows. Since there is no difference +/- β-mercaptoethanol, that lane could be removed to facilitate understanding. Moreover, unlike what is stated, the 2 kDa could not correspond to the 6xHis tag only, which has MW of 840 Da. Is this perhaps tag + TEV cutting site?

Author response:
We thank the reviewer for the suggestion. We have moved the figure to the main text and have updated the figure panels on the recombinant expression of Ena1B

5. An important point that is not addressed in the paper is what is the structure of the ruffles? I understand this is beyond the scope of this paper, but this should be explicit.

Author response:
We agree this is an important lack in our understanding of the Ena fibers. Thus far, our attempts to characterize the ruffles have failed. Our current attempts to identify the ruffles by

tryptic digest and MS analysis have not provided candidate subunits. Also, ruffles are not present in recombinant Ena1B fibers, nor when Ena1A or Ena1C are added in, suggesting they do not comprise the Ena1A or Ena1C subunits, or that they do not self-assemble in vitro.
We have expanded the mention of the ruffles in the discussion in response to the suggestion of the reviewer.

6. The only experiment that is missing in the paper is an important control for Fig S3E. There is no evidence that the antibodies are specific for the different subunits, which should be provided. Otherwise the conclusions drawn from the immunogold data could be wrong. These experiments could also test antibody sensitivity, which would help the authors figuring out whether the absence of Ena1C is due to the low abundance of this protein, or simply due to a sub-par antibody. This could and should be discussed in that part of the text. Also, the immunogold data do not show, as stated on lines 195-196, that "native S-type Enas show a mixed Ena1A and Ena1B composition". They show that both 1A and 1B are subunits, but they do not say that they are in the same pili. It is possible that two different homo-polymeric S-types exist, composed of exclusively of one subunit or the other.

Author response:
We agree with the reviewer that antibody specificity was not explicitly shown and that based on the figures shown, the reader could not exclude a mixed population of Ena1A and Ena1B fibers versus a homogeneous population of mixed Ena1A/Ena1B fibers. Our staining with either antibody is homogeneous, labelling all ex vivo S-Ena rather than a sub-population. Also we like to emphasize that during the cryoEM analysis of ex vivo S-Ena the particle stacks do not split out into Ena1A and Ena1B classes, not at level of 2D nor 3D classification. Therefore, the hybrid Ena1A and Ena1B character of the electron potential maps (Figure S3D) stems from from a mixed composition of the ex vivo S-Ena fibers rather than a mix of homogeneous S-Ena1A and S-Ena1B fibers..
As requested, we now present the evidence that under the experimental conditions used the polyclonal antibodies can discriminate the subunits, and include dot blot and ELISA data that allow the reader to judge the relative binding signal for the three sera (Figure S3).

7. Fig 5. The length results in panel B, for L- and S-type should be separated. Combining them makes this figure confusing because of the vastly different scales for the two types of filaments.

Author response:
We have used a clear color code for the two parts of the figure to further highlight the different length scales for either Ena.

8. The section describing the phylogenetic analysis is unnecessarily long and should be shortened. Also, it could be simplified for the non-experts. For example, the rational link between paragraphs 1 and 2 is unclear at best.

Author response:
We have rephrased and shortened the phylogenetic analysis section to be more accessible to a broad audience and better highlight the novel findings broad by the phylogenetics.

Other comments

1. line 21: the first time the Enas acronym is introduced underline the corresponding letter in

"endospore appendages" to make it obvious what the acronym means. Also, because there are at least two different Enas (S- and L-types) and this papers report the identification and structural characterisation of only one of them, it is necessary to slightly modify the summary to make this point clear. Perhaps call the two Enas ENA-S and ENA-L, and mention that just ENA-S has been characterised in this paper?

Author response:
We have highlighted the point that only S-Ena is structurally characterized in this paper and reviewed the aspect of S- versus L-type Ena nomenclature throughout the manuscript. We settled for 'S-Ena' and 'L-Ena'.

2. line 25: β-augmentation is not a common concept. It would be good to explain in a few words what this actually means.

Author response:
In addition to the review paper that is cited, we now clarify the concept of this protein-protein interaction mode in the text: "In the helical turns, the side by side contact of Ena subunits occurs through β-sheet augmentation, a protein interaction mechanism where an open-edged b-sheet is aligned and extended by a b-sheet or -strand of the interaction partner (Remaut and Waksman, 2006)."

3. line 29: "different eco- and pathotypes" of what? This needs to be spelled out.
Author response:
This has been changed to "Phylogenomic analysis reveals a ubiquitous presence of the ena gene cluster in the B. cereus group which include species of clinical, environmental, and food importance."

4. line 35: "bacteria belonging to the phylum Firmicutes" is too general. Not every Firmicutes produces endospores, far from it actually. Also, Firmicutes should not be in italics.

Author response:
This has been specified and the corrected.

5. lines 50-51: "with multiple micrometers long and a few nanometers wide filamentous appendages" is confusing, as it seems to suggest that there are two types of filaments. Many µm-long filaments, and less nm-wide ones, which is not what the authors intend. Dropping "few" from the above sentence would avoid any confusion.

Author response:
To avoid any confusion we have rephrased to "The surface of Bacillus and Clostridium endospores can also be decorated with multiple micrometers long filamentous appendages, which show a great structural diversity between strains and species (Hachisuka and Kuno, 1976; Rode et al., 1971; Walker et al., 2007)."

6. lines 71-72: The sentence "The genetic identity of the S-type Enas was deduced from the structural model" is difficult to understand. Replace by "The identity of the subunit of S-type Ena was deduced from the structural model".

Author response:
We have modified to "The molecular identity of the S-Ena subunits was deduced from the cryoEM electron potential maps of fibers isolated directly form the endospores, and confirmed by analysis of mutants lacking genes encoding potential Ena protein subunits.".

7. Fig. 1. The order of the panels in this Fig does not reflect the order in which they are mentioned in the text. On line 92, there is mention of "scales pointing down". This is not obvious from this Fig. Moreover, in Fig. 2 the scales seem to be pointing up. The terminal extensions or ruffles ins ENA-S are difficult to see in Fig. 1C. Could the contrast be increased a bit to help visualisation? It should be clearly spelled out in the legend what the distances in Fig. 1C and Fig. 1D insets are. Giving names to the two ENA (see point 1) is particularly important in this part of the text because the results are mainly for ENA-S (e.g. Fig. S1), which is not clearly stated. Also, in the legend to Fig. 1E, its says n=1,023, but there are far more individual data points for the length measurements than for the number of filaments/endospore. Why?

Author response:
We have edited the figure legend to clarify the points made by the reviewer. Unfortunately, because of the high magnification and the low diameter of the ruffles, increasing contrast in the image does not give a better visualization of the ruffles over the background graininess. Regarding the difference in the number of data points in Fig. 1E, this reflects number of individual Enas (1,023) versus number of spores (150) for the length, and spore count plots, respectively. We have edited the legend to avoid any confusion.

8. line 134: considering the resolution of the structure, the use of 5 decimals "3.22937 Å and 31.0338" is not necessary. One decimal seems more than enough. Same applies to numbers on line 172.

Author response:
Indeed, we now round to two decimals.

9. line 145: delete "locus" at the end of this sentence.

Author response:
Locus has been removed.

10. Legend to Fig. 2 is mis-labelled: it is panel E, not F.

Author response:
This has been corrected.

11. line 259. "mutually" is not right the word, replace by "all".

Author response:
This has been changed.

12. line 322: what is the evidence that it has been "misclassified"?

*During search for homologues of the Ena proteins, hits in three specific Streptococcus pneumonia were found (GCA_001161325.1, GCA_001170885.1 and GCA_001338635). As many assemblies in public databases unfortunately suffer from wrongly annotated taxa, a common quality assurance step in genomic microbiology is confirmation of species. To do so, we clustered the three strains with selected Bacillus strains using Mashtree and found that they clustered within the diversity of Bacillus sensu latu. In addition, When applying Tseemans MLST schema on these genomes (*https://github.com/tseemann/mlst*), all three*

*were classified as B. cereus and got a B .cereus MLST profile. The strains had, furthermore, not passed the manual nor the automatic curation in Refseq and Genbank, respectively, due to contamination (*https://www.ncbi.nlm.nih.gov/assembly/GCA_001161325.1*), too large genome size or unconfirmed species (*https://www.ncbi.nlm.nih.gov/assembly/GCA_001170885.1/ and https://www.ncbi.nlm.nih.gov/assembly/GCA_001338635.1*).We therefore concluded that these strains had been misclassified.*

13. lines 427-428: "electron transfer" should be deleted as it is now known that type IV pili of Geobacter are not involved (doi: 10.1016/j.cell.2019.03.029).

Author response:
Whether the extracellular electron transport in Geobacter species is due to conductive type IV pili is indeed still debated. The recent structural studies of OmcS pili (the reference referred to by the reviewer) provides strong support to the existence of conductive pili. Our sentence is not restricted to type IV pili, but gives a generic reference to known functions of the various bacterial pili, which includes electron transfer.

14. legend to Fig. 3: there are more than 19 helical turns in orange on that Fig. unlike what is stated in the legend.

Author response:
To make the 180° turn there are 19, which are numbered. To avoid any confusing we removed the others that were shown.

15. legend to Fig. 4: shouldn't it be "RT-PCR" instead of "PCR" on line 512? Line 517, the line is not "dotted", unlike stated here. Line 518, these are not "whiskers" but simple error bars.

Author response:
This has been changed.

16. line 551: mention what "Nycodenz" is.

Author response:

Nycodenz is an universal density gradient medium. This is explained in the revised methods section.

17. line 562: "kept" should be replaced by "incubated".

Author response:
This has been changed.

18. line 696: "formvr" is a typo.

Author response:
This has been changed.

19. Supplemental information, line 49: correct typo in "loose".

Author response:
This has been changed.

20. Supplemental information, line 58: evidence should be provided that the staining is "specific". See point 6 in Major comments.

Author response:
See our response to point 6.

Referee #2:

This manuscript reports the discovery and structural characterization of a new family of pili that are found on endospores of a large number of Bacillus species and a few other species outside of the Bacillus genus. Termed Enas, for endospore appendages, the authors identified two structurally and compositionally distinct pili, the "staggered-type" (S-type) and "Ladder-like" (L-type). They focus mainly on the S-type pili, solving the structure by CryoEM at a resolution sufficient to identify a short polypeptide that enabled identification of the genetic locus encoding S-type pili. Three proteins, Ena1A, Ena1B, and Ena1C, are the building blocks of S-type pili, where Ena1A and Ena1B appear to randomly assemble into the pilus; how Ena1C contributes to pilus assembly is not yet defined. Both S-type and L-type pili are completely distinct from other pili known to be produced by Gram-positive bacteria, which include sortase-dependent pili and type IV pili that are produced by vegetative cells. Notably, the pilin subunits are tethered together via disulfide crosslinks via a strand donation mechanism reminiscent of that described for Pap/Type I pili of Gram-negative bacteria except that the latter are not disulfide crosslinked. Strikingly purified Ena1A subunits naturally assemble as pili whose structures are highly similar to the S-type pili found on endospores. Overall, this work is well developed and describes structural, compositional, and morphogenetic details of a novel family of pili associated with endospores of Gram-positive bacteria. In fact, these pili were visualized previously but all efforts till now failed in detailed characterization. Thus, the work significantly advances our knowledge of a group of phylogenetically widely distributed pili that likely specifically associate with endospores. The manuscript is very well written and is readily accessible to a broad readership. I have only a few minor comments for the authors to address:

1. Pg. 2, L. 55. Spell out TEM at first use.

Author response:
This has been changed.

2. Fig. 4. It's somewhat surprising that deletions of dedA and 1232 were not analyzed given the proximity of these genes to the ena genes and results of the qRT-PCR? It's a simple extension of the current studies to evaluate whether the encoded proteins also contribute to pilus assembly, especially since the composition of tip fibrils remains unknown.

Author response:
We share the reviewers interest in the possible role of dedA and DUF1293 in Ena assembly. However, we like to point out that these genes are also found in strains lacking the ena genes. Because genetics knockouts in B. cereus are not trivial, we here focused our

attention on the three ena genes. We do have dedA and DUF1293 on the to do list, but are of the opinion that this falls outside the scope of current manuscript.

3. Fig. 2 legend. Reorganize the (A,B) and then (A) and (B) portions of the first sentence. (F) should be (E).

Author response:
We have edited and expanded all figure legends in the manuscript, also in response to the suggestion made by reviewer 1.

4. It would be a nice complement to the PCR data to see levels of 1A, B, and C proteins at the different time points by western blotting. This would validate that transcription correlates with protein synthesis, which is often not the case.

Author response:
This is an excellent suggestion, although complicated by the fact that we have not found conditions to track monomeric Ena1A-C subunits by SDS PAGE & western analysis (we failed to resolved pre-assembled in vitro or ex vivo fibers in SDS-PAGE). We now show a whole cell ELISA tracking Ena subunits in whole cell lysates of B. cereus cultures.

5. Pg. 9, L. 260 & Fig. 5. Why was the Delta *ena1B* mutant complemented with ena1A/1B expression, this results in overproduction of both 1A and 1B. What is the phenotype of transexpressing just 1B? Also, although likely, there is no evidence that transexpression yields overproduction of Ena1A and Ena1B. This should be validated by westerns.

Author response:
We now added additional experiments showing the individual complementations of the ena1A and ena1C knockouts. We have repeatedly tried to compliment the ena1B knockout with a plasmid carrying ena1B as a single gene. Although the plasmid and construct are the equivalent of the single gene constructs for ena1A and ena1C, we have continuously failed to transform this plasmid to B. cereus. The reason for this is unclear. We add a note to this point in the revised manuscript. Western validation is made difficult because of the lack of disassembly of existing Ena fibers. The complementation experiments unequivocally confirm the plasmid-based expression of the complementing subunits, however.

6. In my version of the manuscript, there is no Fig. S5 that corresponds to that referred to in the text, pg. 9. Are Fig. 5 and S5 the same? If so, lettering of the images needs to be revised.

Author response:
This has been corrected.

7. The question of whether the impact on exosporium production and S-type Ena entrapment accompanying Ena1A/1B expression relates to timing or abundance of expression could easily be answered by monitoring phenotypic consequences of gene induction at different times in the growth curve.

Author response:

We agree that how the synthesis and assembly of appendages is coordinated with the assembly of the exosporium layer is an important line for further studies using techniques

such as fluorescence time-lapse microscopy which can illustrate dynamic expression profiles and link them to events in the sporulation process. We have acknowledged this and suggested it as a topic for further research in the discussion part of the revised manuscript. We now do add whole cell ELISA to document the expression of the different Ena subunits in addition to the transcription experiments.

8. Supp. Pg. 1. L. 17. Change 'loose' to 'lose'.

Author response:
This has been corrected.

9. Suppl. Fig. S2. It's interesting that TEV or b-ME treatment have the same effect on Ena1B migration. Explanation?

Author response:
Figure S2 is now upgraded to a main text Figure upon suggestion of Reviewer 1. We have clarified the SDS-PAGE by adding labels of the different species seen, and additional description in the legend.

10. Discussion, Pg. 13, L. 374. There are, however, Gram-positive pili in which the pilin subunits have intramolecular disulfide bonds. This should be mentioned/referenced.

Author response:
Correct, intramolecular disulfides are a quite common stabilization mechanism in different pili. We have adjusted the discussion to make this clear:
"Intramolecular disulfides are important for the structural integrity and assembly of most chaperone/usher, Type IV and several sortase-mediated pili (Bergeron and Sgourakis, 2015; Crespo et al., 2012; Reardon-Robinson et al., 2015). Intermolecular disulfides, however, have not previously been observed. Covalent cross-linking of pilus subunits does form the assembly mechanism underlying sortase-mediated pili in Gram-positives, where the subunits' C-terminal LPXTG motif enables isopeptide bond formation with a conserved lysine in the succeeding subunit (Ton-That and Schneewind, 2004)."

11. Discussion, Pg. 14. L. 407. Can pilus production on vegetative cells be aberrantly stimulated by induction of ena1A/B genes from an inducible promoter? If so, this would allow for the possibility but indicate that pilus production on endospores is controlled at the transcriptional level presumably by signals that remain to be identified.

Author response:
This is an interesting suggestion and a point that we will take up in the study of the assembly pathway and mechanism. In this manuscript, the main focus falls on the molecular identification of Ena as a novel superfamily of pili and on the structural characterization of S-Ena. Although we show that Ena1B will readily self-assemble in vitro, the in vivo assembly pathway is still rather enigmatic. A first question is the location of Ena production, i.e. endospore or mother cell, and how Ena subunits (which lack signal peptides) reach the site of assembly. A second question is whether in vivo, there is a templating structure that localizes and anchors Ena fibers on the endospore. Additionally, the microscopic analysis of L- and S-Enas presented in this study shows that their localization is very different.
Taken together, we are of the opinion that such questions are better answered in a study that systematically addresses in vivo Ena assembly.

12. The discussion is a bit redundant with sections of the Results and could be shortened.

Author response:
We have edited parts of the discussion to include the additional aspects requested by the reviewers. In places the discussion does repeat the main findings presented in the results. We feel this is warranted since it this is contextualized in comparison with other pilus systems.

Referee #3:

The manuscript by Pradhan and colleagues describes the structural and molecular characterization of a novel fiber on the surface of spores generated by Gram-positive organisms. The work is beautifully and carefully done. Some sections of the manuscript are somewhat dense and a bit too descriptive, but apart from that, it was a pleasurable read. There are some glitches, including the absence of what looks like an important figure, as well as the lack of comparisons with other well-studied Gram-positive pili, but in general this manuscript presents data regarding a poorly described bacterial surface appendage, and provides a clear addition to knowledge on such structures. Some more specific comments/questions can be found below.

Readers may not be familiar with the molecular organization of endospores, and a schematic figure that aids in the comprehension of lines 41-50 would also be helpful for the understanding of Fig. 1a.

Author response:
We thank the reviewer for this suggestion and have included such schematic figure as part of Fig. 1.

Are Enas-encoding genes constitutively expressed, generating spores that are always covered in fibers, or is there an expression activation mechanism?

Author response:
We have not studied the regulation of the ena gene cluster other than the RT-PCR transcription analysis that is shown in Figure 5C. We now also add an ELISA-based quantification of Ena1A, Ena1B and Ena1C occurrence in whole cell lysates (Figure 5D). We agree that actual regulatory mechanism is an interesting and important point, but are of the opinion that this better addressed in a dedicated study of the in vivo Ena assembly pathway. See also our response to point 11 of reviewer 2.

Lines 91-92: '... S-type Enas ... give a polar, staggered appearance ... with alternating scales pointing down to the spore surface'. Could this be highlighted directly in the figure to facilitate comprehension?

Author response:
We have clarified Ena orientation relative to the endospore body in Figure 1 and 2.

Lines 102-103: in addition to characterizing fibers biochemically, It could be of interest for authors to characterize the fibers by AFM in order to provide a numerical value regarding strength limitations.

Author response:
We share the reviewers curiosity and interest in performing more detailed analysis of the mechanical properties of the Ena, both in vivo on the spores, as well as the material property

in case of the in vitro fibers. We have initiated such experiments, but are of the opinion that this exceeds the scope of current manuscript.

Lines 138-140: the experiments described in these lines are absolutely remarkable. Authors deduced the sequence of a peptide from a 3.2 Å EM map and bootstrapped their way to the identification of the building block of the fiber itself, and subsequently, characterization of the fiber. This incredible detail could be highlighted at the end of the introduction or in the discussion.

Author response:
We thank the reviewer for his/her enthusiasm on this structure-based molecular identification of the Ena pathway. A point also shared by reviewer 1. In response, we have moved Fig. S2 to the main figures (now Figure 3) and have highlighted this aspect in abstract and main text.

Lines 168-169: authors should clarify on the images what the mean by 'the distal ruffles seen in ex vivo fibers'

Author response:
This has been clarified, both in the figure and figure legend.

Lines 183-185: the only somewhat worrisome aspect of this experiment is that there is no negative control for labeling done with recEna1A or recEna1b sera. It would be of interest to show that their samples are not simply 'sticky', lighting up other unrelated fibers. Is it possible to do this control experiment?

Author response:
The experiments describing the immunolabeling of the ex vivo Ena fibers has been expanded and now includes these controls as part of Figure S2. See also our response to point 6 of reviewer 1.

Line 264: Where is figure S5D?

Author response:
This was mislabeled and has been corrected.

Line 267: the link to this figure does not seem to be correct, and thus the phrases starting from this line and going on towards the end of the paragraph are very difficult to understand.

Author response:
This has been corrected.

Lines 276-344: this section could benefit from shortening & rewriting.

Author response:
This has been rephrased and shortened, also in response to suggestions made by reviewer 1 (point 8).

Lines 423-451: authors comment on the continuing lack of knowledge on the function of the

Ena fibers. Since they have constructed individual Ena knockout strains, would it be possible to do experiments to verify surface adhesion/structure robustness/binding?

Author response:
We agree with the reviewer that following their molecular description, functional studies of the Ena fibers are of top priority. These studies are ongoing and performed in a systematic way, including both S- and type L-Enas. The current results are fragmented and too premature to include in present manuscript. We are of the opinion that such functional studies would be better bundled into a dedicated comprehensive study of Ena function, combining biochemical and biological validation of Ena function.

The Ena fibers described in this work are very elegantly described, and authors have gone to great lengths to characterize these poorly known structures. However, there is an 'uncanny' lack of mention of other pilus systems that have been characterized in detail in Gram-positive bacteria and that show some similarities to the work described here. For example, despite the fact that sortase-mediated pilus assembly was cited, pili from the Gram-positive pathogen Streptococcus pneumoniae, that also display covalent bond formation within subunits, individual domains with beta-sandwich folds, and head-to-tail arrangements with recognition of isolated peptides from adjacent domains, as also shown for the Ena fibers, were not mentioned at all and should be discussed. Many interesting papers describe details of these pili (Hilleringmann EMBO J 2009; Izore Structure 2010; Shaik JBC 2015; Gentile JBC 2011). In addition, some space in the discussion should also be given to type IV pili, that were only superficially mentioned (Berry JBC 2019). This does not diminish the novelty of the work presented in the manuscript, and at the same time highlights details of the plethora of interesting fibers present on the surface of Gram-positive organisms.

Author response:
We have expanded the discussion to make more reference and comparison with other known pilus systems.

Minor remark:

Fig. S2b: it is not clear to this reviewer what the blue boxes are supposed to be highlighting or regrouping. Residues such as Leu and Asp are grouped together, as are Thr, Gly and Asn. Authors should revise the input file of the program employed to generate the figure.

Author response:
We have edited the figure legend to clarify the message brought by the highlighted regions.
These contiguous areas of >50% similarity.

1st Revision - Editorial Decision 11th Mar 2021

Thank you for submitting a revised version of your manuscript. Your revised study has now been seen by all of the original referees, who now broadly support publication of the revised manuscript. Therefore, I would like to invite you to address the remaining editorial issues before I can extend the official acceptance of the manuscript.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I look forward to receiving the final version!

Referee #1:

The revised manuscript by Pradhan et al. has addressed most of my comments on the original version. Although I understand the authors arguments and I tend to think they are right, I am still not convinced that we have a clear demonstration in this paper that the filaments are heteropolymers of Ena1A and Ena1B. I would just suggest to slightly tone down the corresponding statements.

Referee #2:

This revised manuscript has satisfactorily addressed all of my previous concerns, which were relatively minor. The reviewer responses also suggest the same for the other reviewers, but they can comment on this point. I do not have further comments or concerns about this nice piece of work.

Referee #3:

The authors have adequately addressed all of my points.

The authors performed the requested editorial changes.

2nd Revision - Editorial Decision 16th Apr 2021

Editor accepted the manuscript.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Han remaut, Marina Aspholm
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2020-106887

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- ➔ the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ➔ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- ➔ graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- ➔ if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- ➔ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- ➔ a specification of the experimental system investigated (eg cell line, species name).
- ➔ the assay(s) and method(s) used to carry out the reported observations and measurements
- ➔ an explicit mention of the biological and chemical entity(ies) that are being measured.
- ➔ an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- ➔ the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- ➔ a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- ➔ a statement of how many times the experiment shown was independently replicated in the laboratory.
- ➔ definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Where means are presented without statistical tests, N is at least 3, where statistical tests are emplyed N was ≥18. No pre-specified effect size was set.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	YES, where applicable to the statistical test, normality of the data was evaluated using the D'Agostino-Pearson normality test as implemented in graphpad prism 9.
Is there an estimate of variation within each group of data?	NO

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	YES

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Primary antibodies against Ena subunits were generated in this study and their selectivity evaluated (Figure S3). Secondary antibodies conjugated to alkaline phosphatase are from a well characerized commercial source (A3687, Sigma).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Sequences and structural data have been deposited in public databases, with accession numbers declared in the "Data Availability" section.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Dataset have been made available
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA