EMBO reports

Comparison of cable bacteria genera reveals details of their conduction machinery

Leonid Digel, Mads Justesen, Nikoline Madsen, Nico Fransaert, Koen Wouters, Robin Bonné, Lea Plum-Jensen, Ian Marshall, Pia Jensen, Louison Nicolas-Asselineau, Taner Drace, Andreas Bøggild, John Hansen, Andreas Schramm, Espen Bøjesen, Lars Nielsen, Jean Manca, and Thomas Boesen

Corresponding author(s): Thomas Boesen (thb@inano.au.dk)

Review Timeline:

Submission Date:	11th Jul 24
Editorial Decision:	13th Aug 24
Revision Received:	13th Dec 24
Editorial Decision:	16th Jan 25
Revision Received:	29th Jan 25
Accepted:	30th Jan 25

Editor: Achim Breiling

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.)

Dear Dr. Boesen,

Thank you for the submission of your manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees find the study interesting. The referees have several comments, concerns, and suggestions, indicating that need to be addressed to allow publication of the study in EMBO reports. As the reports are below, and all the concerns need to be addressed, I will not detail them further here. It seems referee #3 has seen a previous version of the manuscript at a journal outside of EMBO press. He indicates that the version now submitted to us has improved and her/his previous concerns have been adequately addressed. The referee has some minor remaining points that I also ask you to address during revision.

Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We now request the publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also for EV figures and all those in an Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also: http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams.

9) Please add scale bars of similar style and thickness to microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

10) Please also note our reference format:
http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions and do NOT provide your final manuscript text file with an author contributions section. See also our guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

13) All Materials and Methods need to be described in the main text using our 'Structured Methods' format, which is required for all research articles. According to this format, the Materials and Methods section should include a Reagents and Tools Table (listing key reagents, experimental models, software, and relevant equipment and including their sources and relevant identifiers), uploaded as separate file, followed by a Methods and Protocols section in which we encourage the authors to describe their methods using a step-by-step protocol format with bullet points, to facilitate the adoption of the methodologies across labs. More information on how to adhere to this format as well as downloadable templates (.doc) for the Reagents and Tools Table can be found in our author guidelines (section 'Structured Methods'):

https://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

14) Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements including funding information) - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File (RPF) to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised form of your manuscript when it is ready.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The work described by Digel and co-authors provides a very interesting and scientifically sound observations on cable bacteria. These bacteria are electrically conductive and conduit electricity through periplasmic conductive fibres (PCFs) at centimetre scales. The fibres present very high conductivity and given their length open important avenues for eventual practical applications. The study focuses mainly on single-strain enrichment of the genera Electrothrix and Electronema that allowed the authors to surpass an important bottleneck associate with the used of environmental samples typically containing mix-cultures of cable bacteria, which have impaired through the years to differentiate between intra- and inter-strain variability amongst other key features. The quality of the work and the new findings here reported warrants its publication and seed important routes for future interdisciplinary investigations in the field.

By combining several complementary techniques, the authors show that the electrically conductivity is very different in cable bacteria. This apparently cannot be correlated with the newly identified junction lamellae, which also revealed distinct composition amongst the targeted cable bacteria. Another relevant discover was the observation of the formation of cytoplasmic vesicles originating from the inner membrane. On this topic, I suggest to the authors to develop the possible organization of the putative electron transfer chain components embedded in these structures and how they interplay with the other morphological structures discussed in this manuscript.

Finally, the discovery that iron co-localizes with sulfur and nickel in PCFs, suggest an important role for iron-binding proteins in the conductive properties of cable bacteria. On the other hand, the genomic analysis carried out by the authors seems to exclude the presence of Ni-binding protein in the formation of the PCF. This is a point of debate within the community. Instead, the presence of c-type multiheme cytochromes were revealed from this study. The authors refer to a pentaheme cytochrome with homology to the Geobacter ExtKL and ExtN cytochromes. Given the predicted cellular localization of ExtKL and ExtN cytochromes, the authors should discuss this in more detail, within the context of cable bacteria, the putative role of the pentaheme cytochrome including the rational for the association of this cytochrome with truncated hemoglobin domains in the same species.

In the particular case of the small version of the pentaheme cytochrome (sPHC) its putative role, compared to the structural

homolog pentaheme cytochrome c552, should also be addressed in more detail. In Figure 5, an additional panel containing only the heme groups and respective distances (iron-iron or edge-edge) will be very useful. This is not clear enough from what is presented in panel C.

Referee #2:

This study performs some of the first high resolution imaging using single-isolate cable bacteria cultures, in many cases confirming past observations but under conditions where the data can be conclusively linked to a single organism/genome. A recent observation finding nickel associated with cables is repeated, although quantification of this signal remains elusive. TEM and especially Cryo-ET imaging reveals new features I have not seen elsewhere, such as structures at the cell:cell interface, vesicles, and evidence that 'cables' are made up of smaller ~3 nm parallel fibers. Finally, some genome gazing produces some speculation about cytochromes, perhaps the most notable being their similarity to subunits of Archaeal nanowires. No data is provided or referenced for whether these cytochromes are known to be expressed based on prior data, so these discussions remain at a basic level.

I appreciate the skepticism throughout the discussion and lack of definitive conclusions that often plagues the conductivity field. This work presents new but understandably qualitative information, in a form that others can go out and verify for themselves.

1. P3. It is welcome to see some effort to provide 'controls' for long-bacterium conductivity. But, it is not clear the conductivity of 'non-cable bacteria' can be compared to measurements with cable bacteria. Cable bacteria were fixed to a probe system with carbon paste and a slight vaccum. There are zero additional methods describing the IDAs, but text suggests 'filamentous' bacteria were pipetted onto IDAs and maybe dried (?) and no carbon paste or other approaches were used (?). Provide measurements of cable bacteria tested via the same method or warn readers *in the main text* of differences with the paste/dried/probe systems.

2. P3. Data from 'Rat' and 'Hou', (apparently enrichments of some unknown age), are in the first figure, but all information about them is buried in supplementary. No images are provided to indicate how cross section is calculated, or how similar they are to the RB/GS samples. While they are lab enrichments, data in Fig S1 says it is metagenome sequencing of environmental sediments. Provide accurate descriptions of Rat and Hou (as representative cross sections and on the phylogenetic tree in the main figure/text of the paper).

3. P3. "The measured currents were used to calculate the conductivity of single PCFs, as they are the conductive structures within cable bacteria filaments". This has to read "...as they are hypothesized to be the conductive structures..." -- while it is clearly a reasonable hypothesis, we have been down this road before where something was pointed to as *the conductive thing* and it turned out to be totally wrong.

4. P3. By the same token, correct language in other sentences to reflect this current is being projected into these filaments *mathematically* by some estimate: "The electrical conductivities of the PCFs had the same...' becomes "The calculated electrical conductivities, based on estimates of PCF cross sectional area, had the same...", can correct P7 "The electrical current running through cable bacteria filaments" to "... is **most likely** conducted by..."

P3. Appreciate the honesty in showing instances where samples were below the limit of detection.

5. P3. Elemental/STEM-EDX analysis. It is helpful that this tries to compare and verify with recent studies, and reports similar findings. But, now that a signal can be found, how is a reader supposed to know what a 'strong' signal is? S is a common component of protein, so bands of N and S suggest protein, but everything seems adjusted for the max intensity, so for all one knows there is 100x more S in these images than Ni. There is nothing to compare to. Provide some kind of quantitative information.

The high noise level in the Ni images suggests these are cranked up in sensitivity; without more information it can mean the sensitivity keep changing until we get the image we want. Are we talking sensitivity of 0.1 at%? 10 ppm? What would lead us to think this is a *lot* of Ni or S? Can the ratio of S:N, S:Ni or S:Fe counts be expressed, at least based on the settings used, to show there is an enrichment?

Fig S6. There sure seems to be a lot of Cu and Fe in these samples, based on raw EDS counts which is supposed to correlate with quantity, but this is not discussed. The x-axes are different for B and C vs. A, making it harder to align common peaks.

6. P5. No information is given to support statements like "No putative nickel-sulfur periplasmic fiber candidate proteins were identified from the GS and RB genomes." - what were the criteria? Signal peptides? Tat signals? Binding motifs? Searching for the word "Nickel-Sulfur"? List the Pfam or Interpro motifs or HMM patterns searched for.

7. P6. This statement is false: "The periplasmic c-type cytochrome network of cable bacteria thereby appears to resemble that of

Geobacter sulfurreducens but contains fewer members." There are no homologs of any Geobacter quinone oxidioreductases which are genetically and biochemically shown to be responsible for feeding electrons into the periplasm. However, the ACIII cytochrome looks more like it is part of a novel NrfCD-family quinone oxidioreductase one could speculate is a pmf-dependent link between the quinone and periplasmic pools. These haven't been decribed in Geobacter.

There are no homologs of the most abundant triheme periplamsmic cytochromes also shown to be functional in vivo in Geobacter. The PpcA (triheme)/1996 (dodecaheme) homolog looks to be a hexaheme of similar fold, but there is no direct homolog in Geobacter.

There are no homologs of periplasmic cytochromes proven to be part of functional porin-cytochrome complexes. What is present, beyond a bunch of monoheme (common in many organisms) and diheme (common peroxidases), and possibly catalytic/nitrogen cycling cytochromes (common octaheme family) are some cytochromes that are most interesting in their similarity to the Archaeal nanowire subunits. The ExtIKLMN system is biochemically shown to be an outer membrane porin-cytochrome complex in Geobacter, linked not to anything periplasmic but instead to electron transfer across the outer membrane. The presence of multiple ExtKL-like cytochromes, with their little hydrophobic beta-sheet dangling out, yet no apparent porin/outer membrane component, is very un-Geobacter, highlighting how different the periplasmc network is, rather than similar.

8. It would be immensely helpful to readers who lack access to all the old locus tags and data to check if homologs of any of these proteins are highly abundant or highly expressed in any past transcriptional/proteomic experiments. It would strengthen the case for getting interested in any of these if there was evidence they were 1) present in all or most cable genomes, and 2) were known to be expressed. If it's not a nickel highway and they are nanowires running somewhere in the cell, a lot of protein would be needed.

Referee #3:

I have reviewed this article in a different journal. This draft has improved since the last submission, and my previous concerns have been addressed. I only have one final point:

Under STEM-EDX, Ni, S, and Fe have nearly identical signals. While it's intriguing that the author identified potential cytochrome nanowire homologs, the absence of Ni-S protein candidates raises questions. Were additional elements measured to confirm the abundance of only Ni and S? Additionally, are there any sequence motifs for Ni-S proteins that could be searched for within the genome?

Point-by-point reply to the reviewers EMBOR-2024-59924V1

We thank the Reviewers for constructive critique. We have generally revised the manuscript and below you can find our point-by-point replies to the comments.

Referee #1:

The work described by Digel and co-authors provides a very interesting and scientifically sound observations on cable bacteria. These bacteria are electrically conductive and conduit electricity through periplasmic conductive fibres (PCFs) at centimetre scales. The fibres present very high conductivity and given their length open important avenues for eventual practical applications. The study focuses mainly on single-strain enrichment of the genera Electrothrix and Electronema that allowed the authors to surpass an important bottleneck associate with the used of environmental samples typically containing mix-cultures of cable bacteria, which have impaired through the years to differentiate between intra- and inter-strain variability amongst other key features. The quality of the work and the new findings here reported warrants its publication and seed important routes for future interdisciplinary investigations in the field.

By combining several complementary techniques, the authors show that the electrically conductivity is very different in cable bacteria. This apparently cannot be correlated with the newly identified junction lamellae, which also revealed distinct composition amongst the targeted cable bacteria. Another relevant discover was the observation of the formation of cytoplasmic vesicles originating from the inner membrane. On this topic, I suggest to the authors to develop the possible organization of the putative electron transfer chain components embedded in these structures and how they interplay with the other morphological structures discussed in this manuscript.

Reply: We thank the reviewer for this suggestion! We have now integrated the previously identified putative electron transport chain components from the publication of (Kjeldsen et al, 2019) into the Figure 6, and schematically represented how electrons could be transferred from the electron transport chain, towards the pool of periplasmic cytochromes (pMHCs) and the periplasmic conductive fibers (PCFs). The legend of the Figure 6 was rewritten accordingly.

Finally, the discovery that iron co-localizes with sulfur and nickel in PCFs, suggest an important role for iron-binding proteins in the conductive properties of cable bacteria.

On the other hand, the genomic analysis carried out by the authors seems to exclude the presence of Ni-binding protein in the formation of the PCF. This is a point of debate within the community. Instead, the presence of c-type multiheme cytochromes were revealed from this study. The authors refer to a pentaheme cytochrome with homology to the Geobacter ExtKL and ExtN cytochromes. Given the predicted cellular localization of ExtKL and ExtN cytochromes, the authors should discuss this in more detail, within the context of cable bacteria,

Reply: the Discussion and the Results sections have now been rewritten to expand on the potential roles of ExtKL and ExtN cytochromes. Please see the lines 289-300, and 419-427.

the putative role of the pentaheme cytochrome including the rational for the association of this cytochrome with truncated hemoglobin domains in the same species. In the particular case of the small version of the pentaheme cytochrome (sPHC) its putative role, compared to the structural homolog pentaheme cytochrome c552, should also be addressed in more detail.

Reply: We thank the reviewer for this suggestion! Lines 427-441 now contain a more extensive discussion of the putative roles of these cytochromes.

In Figure 5, an additional panel containing only the heme groups and respective distances (iron-iron or edge-edge) will be very useful. This is not clear enough from what is presented in panel C.

Reply: The Figure 5 and its legend were modified accordingly. The new panel C now contains only the heme groups and iron-iron distances.

Referee #2:

This study performs some of the first high resolution imaging using single-isolate cable bacteria cultures, in many cases confirming past observations but under conditions where the data can be conclusively linked to a single organism/genome. A recent observation finding nickel associated with cables is repeated, although quantification of this signal remains elusive. TEM and especially Cryo-ET imaging reveals new features I have not seen elsewhere, such as structures at the cell:cell

interface, vesicles, and evidence that 'cables' are made up of smaller ~3 nm parallel fibers. Finally, some genome gazing produces some speculation about cytochromes, perhaps the most notable being their similarity to subunits of Archaeal nanowires. No data is provided or referenced for whether these cytochromes are known to be expressed based on prior data, so these discussions remain at a basic level.

Reply: Lines 410-415 now contain additional text and a reference to the previously published expression data. Dataset S1, too, was modified and now contains an extra column, in which cytochromes are linked to the publication of Kjeldsen et al 2019.

I appreciate the skepticism throughout the discussion and lack of definitive conclusions that often plagues the conductivity field. This work presents new but understandably qualitative information, in a form that others can go out and verify for themselves.

1. P3. It is welcome to see some effort to provide 'controls' for long-bacterium conductivity. But, it is not clear the conductivity of 'non-cable bacteria' can be compared to measurements with cable bacteria. Cable bacteria were fixed to a probe system with carbon paste and a slight vaccum. There are zero additional methods describing the IDAs, but text suggests 'filamentous' bacteria were pipetted onto IDAs and maybe dried (?) and no carbon paste or other approaches were used (?). Provide measurements of cable bacteria tested via the same method or warn readers *in the main text* of differences with the paste/dried/probe systems.

Reply: we thank the reviewer for their comment. The required information was now moved from the Supplementary Information document to the Methods section of the main text, lines 557-570. Please see the lines 110-113, which explain the rationale for the use of IDAs to inform the readers.

2. P3. Data from 'Rat' and 'Hou', (apparently enrichments of some unknown age), are in the first figure, but all information about them is buried in supplementary. No images are provided to indicate how cross section is calculated, or how similar they are to the RB/GS samples. While they are lab enrichments, data in Fig S1 says it is metagenome sequencing of environmental sediments. Provide accurate descriptions of Rat and Hou (as representative cross sections and on the phylogenetic tree in the main figure/text of the paper).

Reply: Rat and Hou samples were indeed enrichments of cable bacteria, but were not single-strain enrichments and contained a mix of different *Electrothrix* cable bacteria. We were thus reluctant to insert cross-sections into the main text, because it is hard to justify that they are representative of an enrichment culture containing multiple species.
We have now added two cross-sections from Rat and Hou samples to the new Figure EV1, which will be displayed in the main HTML of the paper in a collapsible format, as examples of what kinds of filaments can be found in those samples. However, they can only be used as rough estimates. The data that we now deposited as Source Data for Figure 1B (numerical data – please see Data Availability section) contains a table that lists size measurements of Hou cable bacteria, which were used to estimate the number of PCFs for individual measurements. The size and the number of PCFs in Rat were reported previously and now the text contains proper citations. Lines 550-556 in the Methods section now contain more details on our calculations.

3. P3. "The measured currents were used to calculate the conductivity of single PCFs, as they are the conductive structures within cable bacteria filaments". This has to read "...as they are hypothesized to be the conductive structures..." -- while it is clearly a reasonable hypothesis, we have been down this road before where something was pointed to as *the conductive thing* and it turned out to be totally wrong.

Reply: the text in the respective places was rewritten accordingly

4. P3. By the same token, correct language in other sentences to reflect this current is being projected into these filaments *mathematically* by some estimate: "The electrical conductivities of the PCFs had the same...' becomes "The calculated electrical conductivities, based on estimates of PCF cross sectional area, had the same...", can correct P7 "The electrical current running through cable bacteria filaments" to "... is **most likely** conducted by..."

Reply: the text in the respective places was rewritten accordingly

P3. Appreciate the honesty in showing instances where samples were below the limit of detection.

5. P3. Elemental/STEM-EDX analysis. It is helpful that this tries to compare and verify with recent studies, and reports similar findings. But, now that a signal can be found, how is a reader supposed to know what a 'strong' signal is? S is a common component of protein, so bands of N and S suggest protein, but everything seems adjusted for the max intensity, so for all one knows there is 100x more S in these images than Ni. There is nothing to compare to. Provide some kind of quantitative information.

The high noise level in the Ni images suggests these are cranked up in sensitivity; without more information it can mean the sensitivity keep changing until we get the image we want. Are we talking sensitivity of 0.1 at%? 10 ppm? What would lead us to think this is a *lot* of Ni or S? Can the ratio of S:N, S:Ni or S:Fe counts be expressed, at least based on the settings used, to show there is an enrichment?

Reply: We thank the reviewer for this comment. To allow for comparison of relative amounts of Ni, S and Fe signals we have put together a new Table 2, which presents relative atomic percentages of these elements. We have to note, however, that STEM-EDX data in Figure 2 present elemental distribution maps and cannot be used for absolute quantification, but we added extra text and references to give the readers a sense of how sensitive the techniques is. Lines 393-398.

Fig S6. There sure seems to be a lot of Cu and Fe in these samples, based on raw EDS counts which is supposed to correlate with quantity, but this is not discussed. The x-axes are different for B and C vs. A, making it harder to align common peaks.

Reply: The x-axis was adjusted based on the reviewer's suggestion (new Figure S5, previously S6). Cu is a present in our STEM-EDX instrument and is not originating from the sample. This point is now written in the main text lines 154-158.

6. P5. No information is given to support statements like "No putative nickel-sulfur periplasmic fiber candidate proteins were identified from the GS and RB genomes." - what were the criteria? Signal peptides? Tat signals? Binding motifs? Searching for the word "Nickel-Sulfur"? List the Pfam or Interpro motifs or HMM patterns searched for.

Reply: Thank you for your comment! To address this point, we put together a new table to Dataset S1, which contains a list of putative nickel-binding proteins. We also added more text to elaborate on how this list was made. Lines 258-267, 399-409, and 483-488

7. P6. This statement is false: "The periplasmic c-type cytochrome network of cable bacteria thereby appears to resemble that of Geobacter sulfurreducens but contains fewer members." There are no homologs of any Geobacter quinone oxidioreductases which are genetically and biochemically shown to be responsible for feeding electrons into the periplasm. However, the ACIII cytochrome looks more like it is part of a novel NrfCD-family quinone oxidioreductase one could speculate is a pmf-dependent link between the quinone and periplasmic pools. These haven't been decribed in Geobacter.

There are no homologs of the most abundant triheme periplamsmic cytochromes also shown to be functional in vivo in Geobacter. The PpcA (triheme)/1996 (dodecaheme) homolog looks to be a hexaheme of similar fold, but there is no direct homolog in Geobacter.

There are no homologs of periplasmic cytochromes proven to be part of functional porin-cytochrome complexes. What is present, beyond a bunch of monoheme (common in many organisms) and diheme (common peroxidases), and possibly catalytic/nitrogen cycling cytochromes (common octaheme family) are some cytochromes that are most interesting in their similarity to the Archaeal nanowire subunits. The ExtIKLMN system is biochemically shown to be an outer membrane porin-cytochrome complex in Geobacter, linked not to anything periplasmic but instead to electron transfer across the outer membrane. The presence of multiple ExtKL-like cytochromes, with their little hydrophobic beta-sheet dangling out, yet no apparent porin/outer membrane component, is very un-Geobacter, highlighting how different the periplasmc network is, rather than similar.

Reply: We thank the reviewer for this well-founded comment. The section has now been rewritten accordingly and the statement "The periplasmic c-type cytochrome network of cable bacteria thereby appears to resemble that of Geobacter sulfurreducens but contains fewer members." was removed to avoid confusion.

8. It would be immensely helpful to readers who lack access to all the old locus tags and data to check if homologs of any of these proteins are highly abundant or highly

expressed in any past transcriptional/proteomic experiments. It would strengthen the case for getting interested in any of these if there was evidence they were 1) present in all or most cable genomes, and 2) were known to be expressed. If it's not a nickel highway and they are nanowires running somewhere in the cell, a lot of protein would be needed.

Reply: Thank you for pointing this out. The Dataset S1 now contains extra columns with the information/references related to the past proteomic/transcriptomic experiments.

Referee #3:

I have reviewed this article in a different journal. This draft has improved since the last submission, and my previous concerns have been addressed. I only have one final point:

Under STEM-EDX, Ni, S, and Fe have nearly identical signals. While it's intriguing that the author identified potential cytochrome nanowire homologs, the absence of Ni-S protein candidates raises questions. Were additional elements measured to confirm the abundance of only Ni and S?

Reply: We thank the reviewer for their comment. To resolve the problem of Ni, S and Fe signals appearing identical in STEM-EDX we have put together a new Table 2, which presents relative atomic percentages of these elements. We have to note, however, that STEM-EDX data in Figure 2 present elemental distribution maps and cannot be used for absolute quantification. Extra text was added to the manuscript to strengthen this point.

Additionally, are there any sequence motifs for Ni-S proteins that could be searched for within the genome?

Reply: Thank you for your comment! To address this point, similar to the question from Reviewer 2, we put together a new table to Dataset S1, which contains a list of putative nickel-binding proteins. We also added more text to elaborate on how this list was made. Lines 258-267, 399-409, and 483-488.

Dear Dr. Boesen,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the report from one of the two referees that I asked to re-evaluate the study, you will find below. As you will see, the referee #1 now fully supports the publication of the study in EMBO reports. Referee #2 was completely unresponsive to my invitations to re-assess the study. However, going through your p-b-p-response, I consider the points of this referee (and also the final point by referee #3) as adequately addressed.

Before I can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please provide a comprehensive final title with not more than 100 characters (including spaces).

- Please provide the abstract with not more than 175 words and written in present tense throughout.

- Please order the manuscript sections like this, using (only) these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Methods - Data availability section - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please make sure that all figure panels (main, EV and Appendix figures) are called out separately and sequentially. Presently, there are no callouts for panels 4D and 4E. Please check.

- Please check again that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main and EV figures). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

If n<5, please show single datapoints for diagrams. Moreover:
- Please note that the box plots need to be defined in terms of minima, maxima, centre, bounds of box and whiskers, and percentile in the legend of figure 1B.
- Please note that information related to n is missing in the legend of figure 1B.

- Please add a proper table of contents (TOC) to the first page of the Appendix file mentioning each item with its corresponding page number. Please remove the legends and mentions of movies and datasets from the Appendix. The Appendix file should only contain the Appendix items and their legends.

- Please name the two datasets 'Dataset EV1' and 'Dataset EV2'. Please update file names, titles, legends and manuscript callouts accordingly. Please provide their legends as a separate tab/sheet in the Excel file, or as a README file in the ZIP folder. Finally, please remove their legends from the Appendix file.

- The movie file needs to be renamed to 'Movie EV1' and the corresponding callouts updated accordingly. Please remove the legend from the and provide it as a README file ZIPped together with the movie file.

- Please add callouts to the reagents and tools table in the methods section.

- The bacterial images shown in Fig. 1A are also shown in Appendix Fig. S6A and S6B. Please clearly mention and explain this re-sue in the respective figure legends.

In addition, I would need from you uploaded separately:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short (!) bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure as separate file that provides a sketch of the major findings (not a data image) in jpeg or tiff format (with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready.

Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have appropriately addressed my concerns and suggestions. In my opinion, the manuscript can be accepted in its current form.

All editorial and formatting issues were resolved by the authors.

Dr. Thomas Boesen
Aarhus University
Department of Molecular Biology and Genetics
Gustav Wieds Vej 10
Aarhus C 8000
Denmark

Dear Dr. Boesen,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://www.embopress.org/page/journal/14693178/authorguide#chargesguide

Should you be planning a Press Release on your article, please get in contact with embo_production@springernature.com as early as possible in order to coordinate publication and release dates.

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

>>> Please note that it is EMBO Reports policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://www.embopress.org/transparent-process#Review_Process

EMBO Press Author Checklist

Corresponding Author Name: Dr. Thomas Boesen
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2024-59924V1

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Not Applicable	

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Materials and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, Figure 1, Table 1, Table 2.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Not Applicable	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Materials and Methods, Figure 1
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Materials and Methods, Figure 1

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	