Peer Review File

Manuscript Title: Structure of *Geobacter* pili reveals secretory rather than nanowire behaviour

Editorial Notes:

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

After years of championing a model requiring adherence to the gospel that Geobacter produced pili made up of only PilA-N with metallic-like conductivity created by aromatic pi-stacking, after multiple demands for retractions and corrections of papers that disagreed with the metallic pili hypothesis, this author presents data to argue the exact opposite of his prior work; that there never were any pili in any of the preparations, that the pili are not very conductive, that the pili are rarely outside the cell, that the pili are made up of two proteins instead of one, and there is no aromatic pi-stacking. This is a very important finding and if confirmed, will change the field of conductive appendages forever. The last decade is regrettable.

The title is not proven and cannot be used as is. There is strong data supporting a novel, surprising, and revolutionary structure for the pilA-N-C pilus. There is some data that without the pilA-N-C genes, other proteins cannot be found outside cells, and it is a compelling hypothesis that I like, but we have a long way to go before we have the proof one is a secretion machine 'evolved' for pushing nanowires out. The fact that the authors are unaware of the standard Gsp T2SS is troubling and further shows the secretion hypothesis should not be the title. The title needs to say what it is; "Structure of heterodimeric pseudopili reveals the Geobacter pilus is nonconductive". The title needs to address the conductive pili hypothesis, that is the only reason to publish this paper.

Because of its importance to the past few papers, the abstract should also mention that these pili do not show any evidence of pi-stacking or aromatic residue interactions.

l53 "Similar to ΔpilA-N cells, ΔpilA-C cells also cannot secrete nanowire-forming OmcS and OmcZ…"..this cites unpublished data from a PhD thesis, from before OmcS and OmcZ were known to form nanowires and before anyone knew how to detect them easily, and the mutants are kanamycin insertions which we know are polar. Caution the reader how weak this data is "Similar to ΔpilA-N cells, less OmcS and OmcZ protein was detected outside ΔpilA-C cells in one study".

Figure 1: (this applies to all immunoblots), part of the problem in this field is the antibodies have been poor and nonspecific. At minimum, the whole immunoblot has to be shown in the Supplemental data. I would not accept a paper that did not show a whole blot somewhere. The whole blot, so we can see if there is nonspecific binding of the antibody. Show the whole blot for 1c and 1d in supplemental.

In the methods you must provide the peptide sequences targeted by the antibodies.

L88 "cells, lacking nanowire-forming cytochromes, expressed PilA-N and PilA-C at elevated intracellular levels" – I don't see evidence of expression (RNA) in this paper. If the evidence is qualitative abundance (more protein on a gel), say that, but there is no quantitative data for expression here.

L92 I cannot find in the methods how the authors know the pilin genes were 'overexpressed'…did they use a promoter with known higher expression than the pilA promoter or did they measure this somehow? A correct term might be "constitutive" or reference the promoter used, but 'overexpressed' implies knowledge of the pilA promoter level which is not given. Don't claim something that isn't measured.

Fig 3; Note in legend that the attachment assay is using glass tubes – this assay is highly dependent on the charge of the material used.

Because of the sensitive nature of this data and the effect of custom scripts on interpretation, please amend the "Code Availability" and "data availability" to clarify the authors will follow the guidelines of the NIH workshop on Reproducibility and Transparency in Structural Biology and/or the 2015 GRC cryo-EM conference that agrees to supply images and relevant metadata needed to reproduce a published reconstruction upon request.

Line 163 "Computational studies found very low conductivity in putative PilA-N" This is a made-up conductivity in a made-up structure… say "Computational studies predicted very low conductivity in a theoretical PilA-N structure which has never been observed in nature"

L176 just because two things are about the same size in AFM cannot "confirm" it is made of a protein. Change the statement "confirming that the PilA-N-C filaments used for conductivity measurements are the same". Or better yet, tell the reader where further work is needed. Think of the poor saps who were told for years they were looking at pili in the metallic papers and it turns out to be wrong. Why would I ever trust your guess at an AFM image now?

L 192 "The T2SS in G. sulfurreducens is incomplete, lacking typical components of the machinery"…this is incorrect, as G. sulfurreducens contains a complete cluster (~GSU0318-GSU0330) containing a classical gsp system. Perhaps the authors are thinking of the oxp cluster? Please explain the reasoning here.

L192 Based on the reference to the oxp/copper protein paper (Mehta), the authors have incorrectly assumed that the oxp system is the only T2SS in Geobacter. There is no data where the actual T2SS was deleted that I know of. Lines 191-194 are wrong. Geobacter has a classic, normal T2SS.

L222 Important: "the PilA-N-C filament remains intracellular"— there is no case of a type 2 secretion/type 4 pilin being stockpiled inside the cell, do the authors mean "remains in the periplasm? The abstract also says the pili remain intracellular. Based on cell fractionation, I can see how it would travel with the inner membrane, but anything in the cytoplasm is on its way to the membrane. Clarify, perhaps the middle ground is "cell associated" vs. "extracellular"

Figure 4: Show the whole blot, at least in supplementary. Cytochrome antibodies are notoriously nonspecific.

Referee #2 (Remarks to the Author):

This paper describes the structure and functions of a heterodimeric pseudopili produced by Geobacter sulfurreducens and propose that it functions as a previously unknown secretion machinery for the extracellular translocation of microbial nanowires that are important for extracellular electron transfer, which plays important roles in globally important environmental phenomena.

The authors isolated type 4 pili like filament from G. sulfurreducens overexpressing PilA-N and

PilA-C, carried out cryoEM structural analysis to analyze its structure at 3.8 Å resolution, built an atomic model and identified that the filament is actually composed of these two proteins PilA-N and PilA-C. The structure showed much closer similarities to that of pseudopili rather than type 4 pili, and various functional assays on bacterial adhesion, twitching motility and electron conductivity confirmed that this PilA-N-C filament is not an electron conductive nanowire but functions as a pseudopili. From the intracellular location of PilA-N and PilA-C in the wildtype strain and a defect in nanowire formation by deletion of genes for these two proteins, the authors propose that a short intracellular filament made of PilA-N and PilA-C works as essential part of a previously unknown nanowire secretion machinery.

This is a solid study with all the experimental works carefully designed and conducted. The manuscript is easy to read and comprehensible. Only problem is that the lines of evidence supporting the proposal is indirect and no insight into the secretion mechanism is given or suggested.

Referee #3 (Remarks to the Author):

This paper describes a cryoelectron microscopy study of polymers formed by the hybrid type IV pilin PilA-N and PilA-C from Geobacter and provides evidence that they may have a role in secretion of cytochromes OmcS and OmcZ. Geobacter and related bacteria produce extracellular filaments that are electrically conductive, and the nature of these filaments has been controversial. There was some evidence that they may be type IV pili, while this group has previously published evidence they are polymers of cytochromes. This work has the potential to reconcile these findings, as it could explain why type IV pilus mutants lack conductivity – they are unable to secrete cytochromes that form nanowires. The accompanying paper showing that OmcZ filaments are thinner than OmcS filaments could explain why PilA-N polymers in the same size range were thought to be the conductive entities. This is an interesting study and the first structural example of a type IV filament formed by a heterodimeric subunit resulting from gene fission, a unique finding. However, there are issues with some of the figures, and some of the findings and conclusions are overstated. In particular, the distinction between type IV pili and type II secretion pseudopili is overstated, as is the novelty aspect (lines 67-69). Secretion of proteins via the type IV pilus system is well established and was first reported for Francisella in 2006 (https://doi.org/10.1111/j.1365-2958.2006.05365.x).

Figure 1 has a number of issues. The figure title (and the section heading on line 70) makes the assumption that these proteins are subunits of pseudopili, which I don't feel the authors have conclusively demonstrated; as mentioned, bona fide type IV pilus systems are capable of protein secretion. Panel a shows a comparison of gene orders between Geobacter and Pseudomonas; the Pseudomonas data are incorrect. The authors should refer to the gene order available through the pseudomonas.com server which compares thousands of genomes. P. aeruginosa has the capacity to make type IVa, IVb, and Tad pili so the authors should clarify those being included in this comparison. Panel b contains multiple errors in species names including P. fluorescens, V. cholerae, M. xanthus. The blot in panel d looks like a single lane rather than 3 distinct samples as indicated by the labeling. It's unclear to me why there is an increase in expression of PilAN/C in the deletion mutants (line 89); are the authors trying to make the case for a feedback loop that increases expression when those proteins are absent? Presumably the stoichiometry between N and C peptides is 1:1 but the detection of PilAN is poor here making this hard to evaluate.

Further, if PilAN/C are expressed on the surface under those conditions, that argues against the contention that they are subunits of pseudopili, which are typically very short, sufficient to span the periplasm of gram negative bacteria. Another feature that struck me as odd for pseudopili was the authors' suggestion that the subunits could be glycosylated based on the additional density in the cryoEM data that could not be accounted for. Subunit glycosylation is considered a means of

stabilizing filaments exposed to environmental extremes, or preventing their recognition by phages or in the case of pathogens, by immune responses. Glycosylation of a pseudopilin is functionally illogical given their predicted intraperiplasmic location. Is there a component encoded by the Geobacter type IV pilus cluster that could be a glycosyltransferase? Line 148 mentions that a C-terminal disulfide bond characteristic of type IV pilins is absent, but there are many examples of fully functional type IV pilins that lack disulfides or have them at other sites in the protomer, so this is weak evidence. Line 156 mentions twitching motility as a key functional aspect of T4P but this is true for only a limited number of species. There are many more that produce type IV pili but don't twitch.

Line 95-6, why would these filaments be easily shed? Is that only when they are overexpressed, or is that also a property of filaments produced in the absence of OmcS/Z?

The region of PilAN that is random coil (between L16 and S25 according to the numbering mentnioned in the text) is different from the disordered region in other assembled pilus filaments, which is typically between P22 and P/G42 – what are the authors thoughts on this?

The authors propose that the glycine residues of the C unit allow for 'flap' movement but provide no mutagenesis data to support such a functional role for these amino acids.

For the heterologous complementation studies, it was not clear to me if the subunits from Pseudomonas or Geobacter uraniireducens were overexpressed in trans, which with the native PilANC prevented secretion of the cytochromes? Line 214 says that OmcS continues to be translocated by these strains but the following sentence says the native subunit is required for translocation. This seems contradictory.

Figure 4 legend should explain what OmcZL vs S are. Why are there two forms of OmcZ here but not in Figure 1d? Figure 4e doesn't make sense to me. What prevents the cytochromes from polymerizing in the periplasm? Are the authors proposing that the cytochromes polymerize on the pilins?? Are the cytochromes released from the cell or do they remain connected? A true type II secretion system pushes substrates out and resets for new rounds, while this arrangement would preclude the system from doing anything else after the first assembly. This needs a more careful explanation.

Extended Figure 2 – based on the legend, I think panels f and g are switched?

Extended Figure 8, there appears to be a lot of density unaccounted for, beside the potential glycosylation?

Referee #4 (Remarks to the Author):

This study addresses the controversial structure and function of PilA of Geobacter sulfurreducens, a protein proposed to form electrically conductive type 4 pili (T4P; e-pili or nanowires). The authors approach this question by a combination of high (close to atomic-level) resolution imaging (CryoEM, TEM, AFM), phylogenetic and biochemical analyses, immunoblotting, mass spectrometry, and functional assessment (conductivity measurements, twitching and adhesion assays) with wildtype and select mutant strains, and arrive at the conclusion that PilA (here called PilA-N) does not form T4P (nanowires) but instead, together with its neighboring gene (here called PilA-C), forms heterodimeric pseudopili and thus acts as a type 2 secretion system (T2SS) of outer membrane cytochromes (OmcS, OmcZ), which in turn form nanowires.

First, this study features an important advance of the field, as the high-quality CryoEM analysis provides the long-awaited detailed structure of PilA in Geobacter, and the surprise discovery of a heterodimer filament. The manuscript is well-structured and well-written. On the other hand, the

interpretation of PilA-N-C as heterodimeric pseudopili that act in a type 2 secretion system for OmcS and OmcZ appears premature and is mostly backed up by indirect indications or by previously published data, interpretations, and hypotheses. Experimental evidence for the key claim of the study, the function of PilA in a T2SS (Figure 4e), is lacking, and the novelty of such a discovery is overstated while some alternative interpretations and previous data are ignored. For example, a recent review (Craig et al., 2019 Nature Reviews Microbiology) emphasized the evolutionary, structural, and functional connections between T4P and the T2SS, including the fact that T4P can serve as secretion systems that transport exoenzymes across the outer membrane; e.g. Dichelobacter nodosus (Han et al., J. Bacteriol. 2007) features T4P that are essential for both type IV fimbrial biogenesis, natural transformation, and protease secretion. Therefore, a double function of PilA (here in OmcZ secretion and "e-pilus" formation) would not be without precedence. Liu et al. (Environm. Microbiol. 2019) described "PilA-C" as pilin chaperone required for the expression of electrically conductive pili; this interpretation is also consistent with the current data but not discussed. Finally, the width of the investigated heterodimer (approx. 6 nm) does not match the width reported repeatedly – including by authors of this study - for e-pili (approx. 3 nm; recently reviewed by Lovley & Walker; Front Microbiol. 2019); this raises the question if "mature" PilA-N pili could have been overlooked here; this should as a minimum be discussed.

Specific comments:

Title: contains 3 overstatements and should be toned down; "pseudopili" is still an interpretation, not a proof; "evolved for" is conceptually flawed (we should avoid teleological statements in science) and the evolutionary aspect is unproven; published evidence (Liu et al., Molecular Microbiology 2019) suggested adaptive evolution of the truncated PilA, selected for conferring Fe(III) reduction capability; "the secretion of microbial nanowires" is not proven either: the authors show impairment in OmcZ excretion but OmcS (the actual nanowire protein) appears still to be secreted.

Abstract, l. 23-27: tone down the impact of the "discovery" for "identifying evolution…and …nanowire assembly architecture"; even if proven, this is neither a novel translocation machinery nor do we learn about nanowire assembly by understanding Omc secretion.

Evolution.
l. 45-69 and Fig. 1A: these data and interpretations are neither novel nor original; Liu et al. (Molecular Microbiology 2019) have recently given an excellent summary of the evolutionary discussion of gene fission/truncation of PilA in Geobacters, incl the work of Liu, Zhan, et al., Environ. Microbiol. 2019; Holmes, Dang, Walker, & Lovley, Microb. Genom. 2016; Shu, Xiao, Yan, & Sun, Front. Microbiol. 2016, where phylogentic trees of both PilA-N and PilA-C were already presented; they have also taken the next step and tested the function of the "original" full-lengths PilA-N-C experimentally.
l. 58-60: structural conservation of T4P head domain (including PilA-C) is not that high: the only highly conserved feature is a 3 stranded β-sheet (see also Fig. 3a).
Fig. 1B and l. 62-65: phylogenetically, the Geobacter PilA lineage forms a subgroup of T4P, a fact well-hidden in the displayed, unrooted tree (where only bootstrap support for the PilA-N-C branch is given, not for its affiliation with either pseudopilins or T4P).
l. 66-69: this hypothesis is not novel or original either, it has already been presented by Wang et al, Cell 2019.

Discovery & structure of novel pseudopili:
l.76-85: this is not an indication for pseudopili, as PilA of T4P are also known to be anchored to the inner membrane as reservoirs for T4P production, so this result is not surprising; in contrast, the absence of PilA-N in the extracellular fractions is unexpected, as PilA has previously been detected (e.g., Tan et al. 2016; Wang et al., 2019).
l. 77 and Fig. 1c, d, e: what is the specificity of the antibodies used, i.e. which epitope do they target? Also, the quality of the Immunoblot images is low, and no size markers are included: it is

difficult to discriminate individual lanes, and the densitometric analysis becomes uncertain.
l. 80-82: GroEL was included as control for the cytoplasmic fraction. What was used as control for an extracellular protein (to prove that the extracellular fraction was reliably sampled)? Could the extracellular PilA-N-C have been lost because it is easily shed into the medium?
l. 99/Methods: which sample(s) was used for cryo-EM? ΔomcS/ΔomcZ strains, or WT with overexpressed PilA-N-C, or both? This is not clear (would it matter?)
Fig. 2f and g could be improved by showing density as mesh or with lower transparency
l. 109-111: indeed excellent agreement (3.8/3.9) and good fit of side chains (at this resolution!) NICE!
l. 116: "random coil" is not the correct term, as these loops have a well-defined structures
l. 123-126: How are these charge reversals explained by the structure? This is not explained in the text, and not inferable from Figs. 2f,g: please extend/include a SI figure.
l. 117-129: have any of the "critical interactions" (electrostatic or hydrophobic, e.g. L47:Y43) been experimentally confirmed or are these just assumptions based on the structure? Please clarify.
l. 132: "A periodic acid-Schiff staining assay suggested this density could be attributed to an O-linked glycosylation (Extended Data Fig. 8b-d)" this is not described in Methods.
l. 135-137: stabilization of PilA-N by the binding of PilA-C could also be taken as argument for the proposed chaperone function of PilA-C (designated Spc, short pilin chaperone, in Liu et al. Environm. Microbiol. 2019). Please discuss.

PilA-N-C vs. T4P comparison:
l. 144-150: while I generally agree that the observed PilA-N-C features (hydrophobic stabilization between neighboring subunits; absence of the α-β loop in the globular head; absence of an "essential" disulfide bridge in the D-region) are more similar to T2SS pseudopili than to T4P, there are two concerns: first, these are only indications, as there is no absolute conservation, and none of these features are mutually exclusive for either pseudopili or T4P; second, in the (likely/unlikely? I cannot judge – but please discuss) case that native PilA-N (3 nm) pili exist, the structure of the observed PilA-N-C (6 nm) pili would have a different significance.
l. 150-153: I do not understand the argument that absence of disulfide bridges (which are more common in extracellular proteins) should be indicative of a pseudopilus: pseudopili are located in the periplasm which is not an intracellular compartment with reducing conditions, but rather an oxidizing environment favorable for disulfide bridge formation.
lines 154-161: I do not understand the rationale for these functional studies (cell adhesion and twitching motility): the proposed function for PilA in G. sulfurreducens is e-pili/nanowire formation, not adhesion or motility. What are you disproving here?
Fig 3b: V9 appears twice in PilA-N-C: the lower V9 should be labelled as E5
Fig 3f: it is unclear for me if the aromatic residues shown here are only from PilA-N (as in the theoretical model, Fig. 3e) or really include those from PilA-C (as indicated in the legend). Please add a closeup with model and density showing the aromatic residues for the combined PilA-N-C to SI Figure S5.

Lack of PilA-N-C nanowire structure & function:
Fig. 3h, i: this figure is virtually identical to Fig. 4f, g, published in Wang et al (Cell 2019), except that only part of the data are shown in Fig. 3h, and Wang 4g show a logarithmic scale: are these really original, new data?
l. 166-167 and l. 179-181: I am puzzled about this reasoning and result: it is stated that the theoretical prediction for PilA "on the order of mS/cm" was only valid "in the presence of a strong oxidizing agent or a high voltage". But the direct measurement (-0.01 to 0.01 V) showed a conductivity of 1 mS/cm. Isn't that unexpected? Or do you reckon the prediction would be different for PilA-N-C (would the PilA-C domain be insulating or conductivity-increasing?)? How should a direct measurement of conductance in PilA-N look?
l. 182-186: The original argument for these observation was that OmcS/OmcZ were necessary for electron transfer to e-pili (reviewed in Lovley & Walker; Front Microbiol. 2019); this is not even mentioned here as alternative explanation?
A role of PilA-N-C in Omc secretion:

l. 198-203 and Fig. 4a: this part is not novel, as it basically reproduces the knock-out experiments of e.g. Liu et al (ISMEJ 2018); there are two interesting points, though: (i) in the present study, OmcS secretion is not completely inhibited by deleting PilA, as judged from the weak band in Fig. 4 (left lower panel); and (ii) when Liu et al (ISMEJ 2018) deleted PilB, the Assembly ATPase, extracellular OmcS (and other cytochrome) levels were unchanged although pilus assembly was inhibited. This is in my view a strong argument against an essential role of PilA-N-C in Omc secretion or more generally in a T2SS.
l. 203-219: also in these experiments, OmcS is apparently translocated, although PilA-N-C is replaced by the PilA of Pa or Gu. First, please show these data. How massive is the OmcS translocation? Even for OmcZ, there seems to be a slight translocation with the Pa-PilA (Figure 4a). This is not mentioned in the text. Furthermore, previous experiments, involving the last author, have also shown that replacing PilA by Pa-PilA does not impair OmcS translocation (Liu et al., AEM2014). So again, these results are not entirely novel or original, nor do they support the hypothesis "that the unique structure of the PilA-N-C filament is critical for proper translocation of OmcS and OmcZ nanowires".
Figure 4e, f: in summary, the model presented here is intriguing but still rather hypothetical, and except for the (beautiful) Cryo-EM structure, the current study has contributed very little novel data to prove those hypotheses.

SI Methods:
(unfortunately without line numbers)
Phylogeny: were site conservation filters used for the PilA phylogeny? The uncertainty of the alignment will otherwise translate into quite uncertain branchings. Please give bootstrap values for all branches.
PilA-N-C purification: please add the recipe for Soerensen's buffer
Immunoblotting: what were the epitopes/antigens selected for immunization of the rabbits?
MS analysis: were all bands visible on the gel? How much protein was loaded? Did you try to analyze the region where PilA-N-C was expected to migrate, just in case?
Subcellular fractionations: how many repetitions were made? What were the controls (antibodies) for intra/extracellular, membrane and periplasmic proteins?

SI Extended data:
Figure S1: for the Geobacter PilA alignment, please indicate the end of PilA-N and the start of PilA-C.
Figures S2, S3, S8: the molecular weights of the PilA preparations seems to differ in the different figures, for PilA-N around 10-12 kDa (instead of 6.6), for PilA-C from 15-25 kDa! Please provide complete images with clear size markers instead of small cut-outs.
Table 1: Ramachandran plot – with only 83.85% "Favored", I recommend to use Isolde, Namdinator, or a similar method for improving Ramachandran statistics of the models based on maps in the 3.8/3.9 Å resolution regime.

Author Rebuttals to Initial Comments:

Point-by-point response to all reviewers' comments. Comments are in bold and revisions to the manuscript are in *italics* here and are marked in red in the revised manuscript.

Reviewer-1:

After years of championing a model requiring adherence to the gospel that Geobacter produced pili made up of only PilA-N with metallic-like conductivity created by aromatic pi-

stacking, after multiple demands for retractions and corrections of papers that disagreed with the metallic pili hypothesis, this author presents data to argue the exact opposite of his prior work; that there never were any pili in any of the preparations, that the pili are not very conductive, that the pili are rarely outside the cell, that the pili are made up of two proteins instead of one, and there is no aromatic pi-stacking. This is a very important finding and if confirmed, will change the field of conductive appendagesforever. The last decade is regrettable.

The title is not proven and cannot be used as is. There is strong data supporting a novel, surprising, and revolutionary structure for the PilA-N-C pilus. There is some data that without the PilA-N-C genes, other proteins cannot be found outside cells, and it is a compelling hypothesis that I like, but we have a long way to go before we have the proof one is a secretion machine 'evolved' for pushing nanowires out. The fact that the authors are unaware of the standard Gsp T2SS is troubling and further shows the secretion hypothesis should not be the title. The title needs to say what it is; "Structure of heterodimeric pseudopili reveals the Geobacter pilus is nonconductive". The title needs to address the conductive pili hypothesis, that is the only reason to publish this paper.

We thank the reviewer for this comment that our finding that the *Geobacter* pilus is nonconductive is sufficient for publication, and we have added this in the title. The editor and other reviewers have asked us to focus on the secretory role of pili. We have therefore performed additional experiments and analyses to demonstrate that *Geobacter* pili show localization, structure and function similar to pseudopili of Type 2 secretion system (T2SS). Akin to T2SS pseudopili, *Geobacter* pili show primarily periplasmic localization, extending to the cell surface only at high protein abundance, having lower stability than T4P, and secreting cytochromes. In addition, *Geobacter* pili also show several structural hallmarks of T2SS pseudopili such as a distinct C-terminal domain, a lack of disulphide bonds and a lack of an F1-E5 salt bridge between N-termini of neighbouring pilins that results in lower stability in pseudopili compared to T4P filaments. Therefore, the periplasmic localization, filament structure and secretory function of *Geobacter* pili are more consistent with pseudopili of T2SS and not T4P filaments.

Therefore, we have revised the title as follows: *Heterodimeric structure of Geobacter pili reveals low conductivity and a role in the secretion of microbial nanowires*

Because of its importance to the past few papers, the abstract should also mention that these pili do not show any evidence of pi-stacking or aromatic residue interactions.

As suggested, we have included this important finding in the abstract as follows: *Individual PilA-N-C filaments lack π-stacking and show conductivity 20,000-fold lower than OmcZ nanowires*

l53 "Similar to ΔpilA-N cells, ΔpilA-C cells also cannot secrete nanowire-forming OmcS and OmcZ…"..this cites unpublished data from a PhD thesis, from before OmcS and OmcZ were known to form nanowires and before anyone knew how to detect them easily, and the mutants are kanamycin insertions which we know are polar. Caution the reader how weak this data is "Similar to ΔpilA-N cells, less OmcS and OmcZ protein was detected outside ΔpilA-C cells in one study".

As suggested by the reviewer, we have revised this sentence as follows: *Similar to ΔpilA-N cells,*

a reduced abundance of OmcS and OmcZ was observed outside of ΔpilA-C cells in one study.

In the revised manuscript, we have also presented data demonstrating that the mutants used in this study do not show a polar effect (Supplementary Fig. 12). RT-PCR showed no effect on the downstream gene expression at the mRNA level for both *ΔpilA-N* and *ΔomcZ* whereas *ΔomcS* showed the effect on the adjacent gene *omcT* as expected (Ref. 14). In addition, we have also complemented *ΔpilA-N, in-trans* with an episomal copy of *pilA-N* and *pilA-C*, that reassembled the PilA-N-C filament. This complementation restored the cytochrome secretion, further confirming the absence of any significant polar effect (Extended Data Fig. 9).

Figure 1: (this applies to all immunoblots), part of the problem in this field is the antibodies have been poor and nonspecific. At minimum, the whole immunoblot has to be shown in the Supplemental data. I would not accept a paper that did not show a whole blot somewhere. The whole blot, so we can see if there is nonspecific binding of the antibody.

Show the whole blot for 1c and 1d in supplemental.

As suggested, we have included controls to show that antibodies are specific (Supplementary Figures 5,6, and 13). In the supplementary materials, we have also included whole immunoblots for every immunoblots presented in the main manuscript and extended data figures.

In the methods you must provide the peptide sequences targeted by the antibodies.

As suggested by the reviewer, we have revised the methods to include the peptide sequence targeted by the antibodies used in this study.

88 "cells, lacking nanowire-forming cytochromes, expressed PilA-N and PilA-C at elevated intracellular levels" – I don't see evidence of expression (RNA) in this paper. If the evidence is qualitative abundance (more protein on a gel), say that, but there is no quantitative data for expression here.

We apologize for the confusion. We have clarified in the revised manuscript as follows: *Both ΔomcS and ΔomcZ cells, lacking nanowire-forming cytochromes, showed elevated abundance of PilA-N and PilA-C*. (Fig. 1d,e and Extended Data Fig. 1a-b)

L92 I cannot find in the methods how the authors know the pilin genes were 'overexpressed'…did they use a promoter with known higher expression than the pilA promoter or did they measure this somehow? A correct term might be "constitutive" or reference the promoter used, but 'overexpressed' implies knowledge of the pilA promoter level which is not given. Don't claim something that isn't measured.

We introduced an *in-trans* inducible expression cassette on a plasmid into WT cells to increase the copy numbers of the *pilA-N* and *pilA-C* genes. The increased protein abundance was demonstrated via immunoblotting with samples normalized to cell mass (Extended Data Fig. 2a). We have revised the text to clarify this further to avoid any confusion.

Fig 3; Note in legend that the attachment assay is using glass tubes – this assay is highly dependent on the charge of the material used.

We have added this clarification to the figure 3 legend as well as in the manuscript text.

Because of the sensitive nature of this data and the effect of custom scripts on interpretation, please amend the "Code Availability" and "data availability" to clarify the authors will follow the guidelines of the NIH workshop on Reproducibility and Transparency in Structural Biology and/or the 2015 GRC cryo-EM conference that agrees to supply images and relevant metadata needed to reproduce a published reconstruction upon request.

As suggested, we have amended the text. We have also deposited all the meta-data and raw images in publicly accessible databases.

Line 163 "Computational studies found very low conductivity in putative PilA-N" This is a made-up conductivity in a made-up structure… say "Computational studies predicted very low conductivity in a theoretical PilA-N structure which has never been observed in nature"

We thank the reviewer for this point. We have revised the manuscript accordingly.

L176 just because two things are about the same size in AFM cannot "confirm" it is made of a protein. Change the statement "confirming that the PilA-N-C filaments used for conductivity measurements are the same". Or better yet, tell the reader where further work is needed. Think of the poor saps who were told for years they were looking at pili in the metallic papers and it turns out to be wrong. Why would I ever trust your guess at an AFM image now?

As suggested by the reviewer, we have revised this sentence to state that *the filament height measured by AFM is consistent with that obtained from the atomic structure*. We have also discussed that AFM can resolve structural features and not just the height. The discovery of atomic structures has now allowed correlation of structural features with morphology observed in high-resolution AFM images, such as axial periodicity with helical pitch of nanowires and longitudinal height with the diameter of filament obtained from the atomic structure (Ref. 2, 11).

Additionally, our mass spectrometry and SDS-PAGE data show that the samples are of high purity PilA-N-C and do not contain any other filament-forming proteins such as OmcS or OmcZ (Extended Data Fig. 1d-f), further indicating that the measured filaments are PilA-N-C.

L 192 "The T2SS in G. sulfurreducens is incomplete, lacking typical components of the machinery"…this is incorrect, as G. sulfurreducens contains a complete cluster (~GSU0318-GSU0330) containing a classical gsp system. Perhaps the authors are thinking of the oxp cluster? Please explain the reasoning here.

L192 Based on the reference to the oxp/copper protein paper (Mehta), the authors have incorrectly assumed that the oxp system is the only T2SS in Geobacter. There is no data where the actual T2SS was deleted that I know of. Lines 191-194 are wrong. Geobacter has a classic, normal T2SS.

We apologize for the confusion. In the revised manuscript, we have clarified that *Geobacter* contains both classical gsp system as well as an incomplete T2SS system, as discussed in detail in Ref. 8. Deletion of genes for the incomplete T2SS system did not affect the translocation of

cytochrome (Ref. 43). We have also discussed that other electricity-generating microorganisms - *Shewanella* species - that use classical T2SS system to secrete cytochromes, do not show any secretion defect in the absence of pili (Ref. 45). In contrast, *Geobacter* pili are unambiguously involved in the secretion of both OmcS and OmcZ nanowires (Fig. 4 and Extended Data Fig. 9).

L222 Important: "the PilA-N-C filament remains intracellular"— there is no case of a type 2 secretion/type 4 pilin being stockpiled inside the cell, do the authors mean "remains in the periplasm? The abstract also says the pili remain intracellular. Based on cell fractionation, I can see how it would travel with the inner membrane, but anything in the cytoplasm is on its way to the membrane. Clarify, perhaps the middle ground is "cell associated" vs. "extracellular".

As suggested, we have clarified our intent in the text by using the descriptor '*periplasmic'*.

Figure 4: Show the whole blot, at least in supplementary. Cytochrome antibodies are notoriously nonspecific.

As suggested by the reviewer, we have shown the entire immunoblots for all main text and extended data figures in Supplementary Figures. The specificity of OmcS and OmcZ antibodies have been tested with *ΔomcS* and *ΔomcZ* whole cell lysates respectively, to demonstrate a lack of non-specific binding at the dilutions/loaded protein amounts used in this study (Supplementary Data Fig. 13).

Referee #2 (Remarks to the Author):

This is a solid study with all the experimental works carefully designed and conducted. The manuscript is easy to read and comprehensible. Only problem is that the lines of evidence supporting the proposal is indirect and no insight into the secretion mechanism is given or suggested.

In the revised manuscript, we have performed additional experiments that show direct evidence for the secretory role of pili (Extended Data Fig. 9). Specifically, we show that the cytochrome secretion could be re-established by the *in-trans* expression of an episomal copy of *pilA-N and pilA-C* in the Δ*pilA-N* mutant, restoring the PilA-N-C filament. We have also provided insights into the secretion mechanism by discussing the pili-based secretion model in detail (Fig. 4).

Referee #3 (Remarks to the Author):

This paper describes a cryoelectron microscopy study of polymers formed by the hybrid type IV pilin PilA-N and PilA-C from Geobacter and provides evidence that they may have a role in secretion of cytochromes OmcS and OmcZ. Geobacter and related bacteria produce extracellular filaments that are electrically conductive, and the nature of these filaments has been controversial. There was some evidence that they may be type IV pili, while this group has previously published evidence they are polymers of cytochromes. This work has the potential to reconcile these findings, as it could explain why type IV pilus mutants lack conductivity – they are unable to secrete cytochromes that form nanowires.

The accompanying paper showing that OmcZ filaments are thinner than OmcS filaments could explain why PilA-N polymers in the same size range were thought to be the conductive entities.

This is an interesting study and the first structural example of a type IV filament formed by a heterodimeric subunit resulting from gene fission, a unique finding.

However, there are issues with some of the figures, and some of the findings and conclusions are overstated.

We have revised all the figures as per the reviewer's suggestion and have rewritten the text to avoid any appearance of overstatement.

In particular, the distinction between type IV pili and type II secretion pseudopili is overstated, as is the novelty aspect (lines 67-69).

As suggested, we have rewritten this text to avoid overstatement by clarifying that the main difference between type IV pili (T4P) and T2SS pseudopili is localization and function with some key structural differences. T4P need to be present on the bacterial surface to carry out all their functions whereas pseudopili remain periplasmic and secrete proteins. We have performed additional experiments and analysis to demonstrate that *Geobacter* pili show hallmarks of localization, structure, and function similar to T2SS pseudopili. Akin to pseudopili, *Geobacter* pili show primarily periplasmic localization, extending to the cell surface only at high protein abundance, having lower stability than T4P, and secreting cytochromes. In addition, *Geobacter* pili also show several structural hallmarks of T2SS pseudopili such as distinct a C-terminal domain, a lack of disulphide bonds and a lack of an F1-E5 salt bridge between N-termini of neighbouring pilin that results in lower stability in pseudopili compared to T4P filaments. Furthermore, replacing *Geobacter* pili with T4P does not fully restore the nanowire secretion (Fig. 4c). Therefore, the periplasmic localization, filament structure and secretory function of *Geobacter* pili are more consistent with pseudopili of T2SS and not T4P filaments.

All our structural, functional, and localization studies thus indicate that although *Geobacter* pili are formed by the T4P system, they are similar to T2SS pseudopili, thus suggesting a new class of filaments not previously described with unique heterodimeric structure and primarily periplasmic localization. Akin to T2SS pseudopili, these PilA-N-C filaments are directly involved in cytochrome secretion (Fig. 4, Ext. Data Fig. 9). PilA-N-C filaments protrude from the cell surface only when the abundance of PilA-N and PilA-C is artificially high which is also a hallmark of T2SS pseudopili (Ref. 8,26 and 27). In addition to this functional similarity, PilA- N-C filaments show several structural similarities to T2SS pseudopili such as a lack of a disulphide bridge which is critical for T4P function and has been used to distinguish between T4P and pseudopilins [Ref. A. Filloux, Biochimica et Biophysica Acta **1694** 163–179 (2004)]. We have also highlighted the unique structural elements of PilA-N-C filaments such as their heterodimeric subunits and a lack of a salt bridge between neighbouring pilin subunits which is critical for T4P assembly. To avoid any confusion between *Geobacter* pili and T2SS pseudopili, we have avoided referring to *Geobacter* pili as pseudopili in the revised manuscript.

Secretion of proteins via the type IV pilus system is well established and was first reported for Francisella in 2006 (https://doi.org/10.1111/j.1365-2958.2006.05365.x).

We have now discussed this paper in the revised manuscript (Ref. 48) and note that the secretion of proteins by T4P was reported even before this paper (Kirn et al. Mol. Micro. 49, 81, 2003). However, there are important differences between these previous reports of T4P-based secretion and *Geobacter* PilA-N-C filament-based secretion. First, the unique heterodimeric structure has never been reported before to our knowledge. Second, *Geobacter* pili are unlike other T4P as evidenced by the localization and functional experiments in our manuscript (Figures 1, 3). Finally, T4P-based secretion requires pilus filaments serving as a template for proteins to be secreted and thus requires the filaments to be displayed outside for secretion (Ref. 49). In contrast, *G. sulfurreducens* cells do not appear to display pili on the surface under physiological conditions and pili remain periplasmic during cytochrome secretion, suggesting that pili function

primarily as a secretion system (Figures 1, 4). Furthermore, when *Geobacter pilA*-N was replaced by T4P-forming *Pseudomonas pilA*, secretion of OmcS was restored but the secretion of OmcZ nanowires was still impaired (Fig. 4c). These studies show that *Geobacter* pili are structurally and functionally distinct from T4P. We have discussed this in the revised manuscript.

Figure 1 has a number of issues. The figure title (and the section heading on line 70) makes the assumption that these proteins are subunits of pseudopili, which I don't feel the authors have conclusively demonstrated; as mentioned, bona fide type IV pilus systems are capable of protein secretion.

As suggested, we have changed the title and have avoided referring to Geobacter pili as pseudopili. However, we note that our localization and functional studies demonstrate that *Geobacter* PilA-N-C filaments cannot function similar to type IV pili because they are not surface-displayed under physiological conditions (Fig. 1) and yet are still capable of secreting cytochromes (Fig. 4). *Geobacter* pili are expressed on the bacterial surface only when the subunits are overproduced, including when cytochromes are deleted (Fig. 1). In addition to localization and function akin to T2SS pseudopili, *Geobacter* pili show several structural features that are also hallmarks of pseudopili and not T4P such as a unique C-terminal domain structure, a lack of disulphide bonds, and a lack of an F1-E5 salt bridge. All of these features yield lower stability in *Geobacter* pili compared to T4P, making Geobacter pili more prone to degradation than T4P (Extended Data Fig. 7). The observed lower stability and propensity to degradation is also observed for T2SS pseudopili (Cisneros et al. *Mol. Micro.* 86, 805, 2012). We have also performed additional experiments that further demonstrate the direct role of PilA-N-C filaments in the secretion of cytochromes (Extended Data Fig. 9). Therefore, the localization, structure and function of *Geobacter* pili is more consistent with T2SS pseudopili than with T4P filaments. However, we have avoided referring to *Geobacter* pili as pseudopili to avoid any confusion between T2SS and T4P systems that form *Geobacter* pili.

Panel a shows a comparison of gene orders between Geobacter and Pseudomonas; the Pseudomonas data are incorrect. The authors should refer to the gene order available through the pseudomonas.com server which compares thousands of genomes.

We thank the reviewer for pointing this and we have corrected a typographical error in this figure to clarify that the illustrated comparison is to *Myxococcus xanthus* genes, which have been included to remain consistent with previous publications discussing *Geobacter* pili (Ref. 1).

P. aeruginosa has the capacity to make type IVa, IVb, and Tad pili so the authors should clarify those being included in this comparison.

We have now clarified this in the manuscript as well as in the figure 3 title, stating we have only used type IVa pili for comparison.

Panel b contains multiple errors in species names including P. fluorescens, V. cholerae, M. xanthus. The blot in panel d looks like a single lane rather than 3 distinct samples as indicated by the labeling.

We thank the reviewer for pointing out these errors. As suggested, we have corrected these figure panels with revised figures and new gels.

It's unclear to me why there is an increase in expression of PilAN/C in the deletion mutants (line 89); are the authors trying to make the case for a feedback loop that increases expression when those proteins are absent?

As suggested by the reviewer, a feedback loop is a likely mechanism for increased abundance of PilA-N/C proteins in cytochrome mutants. Previous studies have shown that the cytochrome expression in *G. sulfurreducens* is regulated by the PilS-PilR two-component system (Juarez et al. *J. Mol. Microbiol. Biotechnol.* **16**, 146, 2009), likely through autophosphorylation of PilR, which switches PilS into a kinase state to upregulate protein expression (Kilmury and Burrows, *PNAS* **113**, 6017, 2016). We have previously found that evolved strains show truncation in the *pilR* gene, which is a response regulator known to cause overexpression of nanowire-forming cytochromes (Summers et al. *Science* **330**, 1413, 2010). However, defining a detailed mechanism requires further studies.

Presumably the stoichiometry between N and C peptides is 1:1 but the detection of PilAN is poor here making this hard to evaluate.

As suggested by the reviewer, we have now performed additional experiments (Fig. 1c-e) that clarifies the 1:1 stoichiometry between PilA-N and PilA-C in agreement with the atomic structure (Fig. 2). We have also found similar 1:1 intensity in the purified filament preparation (Extended Data Fig. 1d).

Further, if PilAN/C are expressed on the surface under those conditions, that argues against the contention that they are subunits of pseudopili, which are typically very short, sufficient to span the periplasm of gram negative bacteria.

We show that PilA-N and PilA-C are not expressed on the surface in WT cells under nanowire-producing conditions (Fig. 1c, e, Ext. Data. Fig. 1, Supplementary Data Fig. 2). PilA-N-C filaments are displayed on the surface only when their constituent proteins' abundance is artificially increased (Fig. 1d-e and g, Ext. Data. Fig. 2). This behaviour is consistent with previous studies concluding that T2SS pseudopili are observed on the cell surface only when expression of pseudopilins is artificially increased (Ref. 8, 26 and 27). In contrast, display of T4P filaments on the bacterial surface is essential for their functions, including secretion [Ref. 48].
This important distinction between pseudopili and T4P further demonstrates that PilA-N-C filaments do not function like T4P and are functionally similar to pseudopili.

Another feature that struck me as odd for pseudopili was the authors' suggestion that the subunits could be glycosylated based on the additional density in the cryoEM data that could not be accounted for. Subunit glycosylation is considered a means of stabilizing filaments exposed to environmental extremes, or preventing their recognition by phages or in the case of pathogens, by immune responses. Glycosylation of a pseudopilin is functionally illogical given their predicted intraperiplasmic location.

We appreciate this comment, although we would like to note the suggestion that only extracellular proteins are glycosylated is not supported by the literature. Many periplasmic proteins are glycosylated for certain regulatory functions such as protein-protein interactions and protein recognition [see e.g., Vorkapic et al., *Frontiers in microbiology*, **10**, 2780 (2019)]. As discussed in the previous response, our localization studies demonstrate unambiguously that PilA-N-C filaments remain intracellular under wild-type and nanowire-producing conditions. We have clarified the text to state that although extracellular pili are glycosylated, there are multiple precedents for glycosylation of intracellular and periplasmic proteins.

Is there a component encoded by the Geobacter type IV pilus cluster that could be a glycosyltransferase?

As suggested by the reviewer, GSU1498 (the downstream gene of PilA-C) could be a potential glycosyltransferase. It is homologous to TpfW from *P. aeruginosa* strain PA17, which had been shown to add D-arabinofuranose to *Pseudomonas* pilin [Harvey et al. *Nature Microbiology*, **3**, 47-52 (2018)]. GSU1498 belongs to Xap cluster, which contains other genes that have been shown to be critical for extracellular polysaccharides [Rollefson et al. *Journal of Bacteriology*, **193**, 1023-1033 (2011)].

Line 148 mentions that a C-terminal disulfide bond characteristic of type IV pilins is absent, but there are many examples of fully functional type IV pilins that lack disulfides or have them at other sites in the protomer, so this is weak evidence. Line 156 mentions twitching motility as a key functional aspect of T4P but this is true for only a limited number of species. There are many more that produce type IV pili but don't twitch.

We have further clarified this in the revised manuscript, stating that the purpose of these comparisons is to emphasize that PilA-N-C filaments do not show any behaviours that are characteristic of T4P. These filaments are not displayed on the surface and did not show any involvement in bacterial adhesion, twitching motility or biofilm formation. Despite having some of the hallmarks of T4P systems (e.g., retraction ATPase PilT and pilus tip protein PilY1), *Geobacter* pili show hallmarks of localization, structure and function similar to Type 2 secretion pseudopili as described earlier. We have also clarified this further in the revised manuscript.

Line 95-6, why would these filaments be easily shed? Is that only when they are overexpressed, or is that also a property of filaments produced in the absence of OmcS/Z?

We thank the reviewer for pointing this out. In contrast to T4P as well as nanowire forming cytochromes OmcS and OmcZ, which are tightly bound to cells, surface-displayed PilA-N-C filaments are indeed loosely bound to cells and are released very easily to the media under all conditions in which they were observed. In the revised manuscript, we have included additional experiments demonstrating that PilA-N-C filaments are less stable and are more prone to degradation than T4P (Extended Data Fig. 7). Such low stability and propensity to degradation have also been observed for T2SS pseudopili. These filaments appear to have been evolved to be periplasmic and do not form robust surface-displayed pili.

The region of PilAN that is random coil (between L16 and S25 according to the numbering mentnioned in the text) is different from the disordered region in other assembled pilus filaments, which is typically between P22 and P/G42 – what are the authors thoughts on this?

The random coil region is formed because the helix must melt in this region, facilitated by helix-breaking residues, for the PilA-N subunits to pack in the pilus. As PilA-N-C structure is substantially different than other pili, we have now discussed in the revised manuscript that this region in PilA-N-C is slightly different than that in other T4P, where it typically ranges from G14 to P22 in *Pseudomonas* and *Neisseria* and could extend beyond P22 in *Thermus thermophilus* species [Ref. Neuhaus et al. *Nature Communications*, 11, 1-13 (2020)].

The authors propose that the glycine residues of the C unit allow for 'flap' movement but provide no mutagenesis data to support such a functional role for these amino acids.

As per the suggestion by the editor and reviewers, we have removed this text to keep the focus on

how the structure of *Geobacter* pili explains their lack of conductivity and role in secretion of microbial nanowires.

For the heterologous complementation studies, it was not clear to me if the subunits from Pseudomonas or Geobacter uraniireducens were overexpressed in trans, which with the native PilA-N-C prevented secretion of the cytochromes?

We have clarified in the methods that the heterologous expression was not constructed *in-trans*, but rather incorporated target genes into the genome in *ΔpilA-N* background (Ref. 46).

Line 214 says that OmcS continues to be translocated by these strains but the following sentence says the native subunit is required for translocation. This seems contradictory.

We have clarified in the revised manuscript that the unique PilA-N-C filament structure is essential for the translocation of OmcZ nanowires, while the translocation of OmcS can be restored as long as any T4P are present as reviewed in Ref. 49.

Figure 4 legend should explain what OmcZL vs S are. Why are there two forms of OmcZ here but not in Figure 1d?

We have now clarified in the legend and revised manuscript that OmcZ exists in two forms – an extracellular, small form (30 kDa) which assembles into nanowires (Ref. 5) and a periplasmic, large form (50 kDa). We have included both these forms in Fig. 4 to demonstrate that OmcZ remains periplasmic in the absence of PilA-N-C filament and that the lack of OmcZ nanowires is not due to low protein abundance, but rather is caused by a translocation defect in the absence of PilA-N-C filaments. Both of these OmcZ forms are absent in the Δ*omcZ* mutant shown in Fig. 1d.

Figure 4e doesn't make sense to me. What prevents the cytochromes from polymerizing in the periplasm? Are the authors proposing that the cytochromes polymerize on the pilins?? Are the cytochromes released from the cell or do they remain connected? A true type II secretion system pushes substrates out and resets for new rounds, while this arrangement would preclude the system from doing anything else after the first assembly. This needs a more careful explanation.

We apologize for the confusion and have now further revised the model and text to clarify that the anchoring point of cytochrome nanowires can be at a different location and they do not need to polymerize over the pilins (Fig. 4e). Our experiments suggest that the PilA-N-C filament is primarily involved in pushing the nanowires out of the cell (Extended Data Fig. 9).

Extended Figure 2 – based on the legend, I think panels f and g are switched?

We have now corrected the legend in this figure (now Extended Data Fig. 1).

Extended Figure 8, there appears to be a lot of density unaccounted for, beside the potential glycosylation?

We have now revised the figure, with all coordinates filled, to clarify that all the density is accounted for except the apparent glycosylation of Ser 94 (Extended Data Fig. 6).

Referee #4 (Remarks to the Author):

This study addresses the controversial structure and function of PilA of Geobacter sulfurreducens, a protein proposed to form electrically conductive type 4 pili (T4P; e-pili or nanowires). The authors approach this question by a combination of high (close to atomic-level) resolution imaging (CryoEM, TEM, AFM), phylogenetic and biochemical analyses, immunoblotting, mass spectrometry, and functional assessment (conductivity measurements, twitching and adhesion assays) with wildtype and select mutant strains, and arrive at the conclusion that PilA (here called PilA-N) does not form T4P (nanowires) but instead, together with its neighboring gene (here called PilA-C), forms heterodimeric pseudopili and thus acts as a type 2 secretion system (T2SS) of outer membrane cytochromes (OmcS, OmcZ), which in turn form nanowires.

First, this study features an important advance of the field, as the high-quality CryoEM analysis provides the long-awaited detailed structure of PilA in Geobacter, and the surprise discovery of a heterodimer filament. The manuscript is well-structured and well-written.

On the other hand, the interpretation of PilA-N-C as heterodimeric pseudopili that act in a type 2 secretion system for OmcS and OmcZ appears premature and is mostly backed up by indirect indications or by previously published data, interpretations, and hypotheses.

We apologize for the confusion. Pseudopili were previously found only in T2SS whereas our study is the first to report pseudopili-like structure and secretion function for proteins that are completely independent of T2SS. We have now clarified this further in the revised manuscript by removing the term pseudopili to avoid any confusion.

In the revised manuscript, we have expanded on our localization, structural and functional studies demonstrating that PilA-N-C filaments are similar to T2SS pseudopili. Akin to T2SS pseudopili, *Geobacter* pili show primarily periplasmic localization, extending to the cell surface only at artificially high protein abundance, having lower stability than T4P, and secreting cytochromes. In addition, *Geobacter* pili also show several structural hallmarks of T2SS pseudopili such as distinct a C-terminal domain, a lack of disulphide bonds and a lack of an F1- E5 salt bridge between N-termini of neighbouring pilins that result in lower stability in pseudopili compared to T4P filaments. Therefore, the periplasmic localization, distinct structure and secretory function of *Geobacter* pili are consistent with T2SS pseudopili thanT4P filaments.

Experimental evidence for the key claim of the study, the function of PilA in a T2SS (Figure 4e), is lacking, and the novelty of such a discovery is overstated while some alternative interpretations and previous data are ignored.

In the revised manuscript, we have performed additional experiments that show direct evidence for the secretory role of PilA-N-C filaments (Extended Data Fig. 9). Specifically, we have complemented *ΔpilA-N* strain with an episomal copy of *pilA-N* and *pilA-C* that reassembled the the PilA-N-C filament and restored the secretion of both OmcS and OmcZ nanowires. We have also revised the model and have provided insights into the secretion mechanism by discussing the pili-based secretion model in further detail (Fig. 4).

For example, a recent review (Craig et al., 2019 Nature Reviews Microbiology) emphasized the evolutionary, structural, and functional connections between T4P and the T2SS, including the fact that T4P can serve as secretion systems that transport exoenzymes across the outer membrane; e.g. Dichelobacter nodosus (Han et al., J. Bacteriol. 2007) features T4P that are essential for both type IV fimbrial biogenesis, natural transformation, and protease secretion.

We have discussed this recent review in the revised manuscript (Ref. 50) as well other studies of secretion based on T4P (Ref. 48-49). However, there are important differences between these previous reports of T4P-based secretion and *Geobacter* PilA-N-C filament-based secretion. First, the unique heterodimeric structure has never been reported before to our knowledge. Second, *Geobacter* pili are unlike other T4P as evidenced by the localization and functional experiments in our manuscript (Fig. 1, 3). Finally, T4P-based secretion requires pilus filaments serving as a template for proteins to be secreted and thus requires the filaments to be displayed outside for secretion (Ref. 49). In contrast, *G. sulfurreducens* cells do not display pili on the surface under physiological conditions and pili remain periplasmic during cytochrome secretion, suggesting that pili function primarily as a secretion system (Figures 1, 4). Furthermore, when *Geobacter pilA*-N was replaced by T4P-forming *Pseudomonas pilA*, secretion of OmcS was restored but the secretion of OmcZ nanowires was still impaired (Fig. 4c). Therefore, *Geobacter* pili are structurally and functionally distinct from T4P. We have discussed this in the revised manuscript.

One of the central findings of our study is that under nanowire-producing conditions, PilA-N-C do not produce surface-displayed filaments and are required for the secretion of cytochrome nanowires. Since 2005, we and others had thought that *Geobacter* pili are T4P-based nanowires (Ref. 1). However, our new structural, localization and functional studies show that PilA-N-C filaments are not responsible for electron conduction by *G. sulfurreducens* nanowires and do not form surface-displayed pili like typical T4P biogenesis systems despite sharing features associated with T4P (e.g., retraction ATPase PilT and pilus tip protein PilY1). Instead, they behave like T2SS and show structural hallmarks of pseudopili. We agree that PilA-N-C filaments lie along a continuum between T4P or T2SS and cannot strictly be classified as either (Fig. 1b, Supplementary Fig. 1b). We have further clarified this in the revised manuscript.

Therefore, a double function of PilA (here in OmcZ secretion and "e-pilus" formation) would not be without precedence.

The hypothesis of e-pilus function is based on a theoretical model of a PilA-N-only filament (Fig. 3e in the manuscript, Ref. 36). There is no direct evidence that such a filament exists under natural conditions (Ref. 11). We further show that there is no evidence of any PilA-N-only filament even when *Geobacter* pili filaments are observed. Our studies unambiguously demonstrate that *Geobacter* PilA-N-C filaments are not electrically conductive and thus cannot function as e-pili. Furthermore, we have shown that *Geobacter* does not even produce surface- displayed pili under the previously reported nanowire-producing conditions, unless the abundance of the pilin subunits is artificially increased as occurs in the absence of nanowire- forming cytochromes (Fig. 1). We have further clarified this in the revised manuscript.

Liu et al. (Environm. Microbiol. 2019) described "PilA-C" as pilin chaperone required for the expression of electrically conductive pili; this interpretation is also consistent with the current data but not discussed.

We had discussed this work (Ref. 24, Ref. 25 in the original manuscript). However, the interpretation presented in that work is not consistent with the structure of *Geobacter* pili or our localization data. Liu et al. claimed that PilA-C are not part of the filament and *indirectly* help translocation of putative PilA-N-only nanowires to the bacterial surface. In contrast, we show that

there are no PilA-N-containing filaments on bacterial surfaces under nanowire-producing conditions (Fig. 1 and Supplementary Data Fig. 2) and even under artificial conditions that extend pili to the cell surface, these filaments show very low conductivity, 20,000-fold lower than cytochrome OmcZ nanowires. We also show that PilA-C plays a *direct* role in the structure of *Geobacter* pili by forming the outer surface (Fig. 2) that protects hydrophobic PilA-N from the environment (Extended Data Fig. 5).

Finally, the width of the investigated heterodimer (approx. 6 nm) does not match the width reported repeatedly – including by authors of this study - for e-pili (approx. 3 nm; recently reviewed by Lovley & Walker; Front Microbiol. 2019); this raises the question if "mature" PilA-N pili could have been overlooked here; this should as a minimum be discussed.

In the revised manuscript, we have directly compared the data by the Lovley group about 3-nm pili-like filaments (Ref. 4) with our cryo-EM analysis of such filaments (Extended Data Fig. 8). We show that these filaments are made up of DNA and not protein. They do not show any structural features similar to any previously described pili. We also discuss that any hypothetical PilA-N-only filament would be significantly less stable than the PilA-N-C filament and thus unlikely to assemble under natural conditions due to its highly hydrophobic surface (Extended Data Fig. 5b). In addition, we have discussed in the revised manuscript that without an atomic-resolution structure of a *Geobacter* filament composed solely of PilA-N, there seems to be no conclusive proof that *Geobacter* can make such a filament. Furthermore, conduction along the length of a single bona fide PilA-N filament has not been demonstrated. Finally, we have also discussed the comparison with synthetic proteins containing π-stacking of aromatic side chains that mimic hypothetical PilA-N filament (Ref. 37). Our conductivity measurements and theoretical studies show very low conductivity (~1 μS/cm) due to π-stacking of aromatic chains.

Title: contains 3 overstatements and should be toned down; "pseudopili" is still an interpretation, not a proof; "evolved for" is conceptually flawed (we should avoid teleological statements in science) and the evolutionary aspect is unproven; published evidence (Liu et al., Molecular Microbiology 2019) suggested adaptive evolution of the truncated PilA, selected for conferring Fe(III) reduction capability;

As suggested, we have revised the title and the text. To avoid confusion with T2SS-based pseudopili, we have removed this term. To bring the focus to lack of nanowire behaviour, we have also removed the text about evolution. However, there are important differences between our work and that by Liu et al. (Ref. 47) as discussed in the later response.

"the secretion of microbial nanowires" is not proven either: the authors show impairment in OmcZ excretion but OmcS (the actual nanowire protein) appears still to be secreted.

OmcZ is also actual nanowire protein that forms 1000-fold more conductive nanowires than OmcS (Ref. 5). This work was provided as a related manuscript with the original submission. Deletion of *pilA*-N inhibits secretion of both OmcS and OmcZ nanowires (Fig. 4a,b). In the revised manuscript, we have performed additional experiments that show direct evidence for the secretory role of filaments (Extended Data Fig. 9). Specifically, we have complemented *ΔpilA-N in-trans* with an episomal copy of *pilA-N* and *pilA-C*, restoring the PilA-N-C filament and the secretion for both OmcS and OmcZ nanowires. Furthermore, when *Geobacter pilA*-N was replaced by T4P-forming *Pseudomonas pilA*, secretion of OmcS was restored but the secretion of

OmcZ nanowires was still impaired (Fig. 4c). All these studies show that PilA-N-C filaments are directly involved in the secretion of microbial nanowires.

l. 45-69 and Fig. 1A: these data and interpretations are neither novel nor original; Liu et al. (Molecular Microbiology 2019) have recently given an excellent summary of the evolutionary discussion of gene fission/truncation of PilA in Geobacters, incl the work of Liu, Zhan, et al., Environ. Microbiol. 2019; Holmes, Dang, Walker, & Lovley, Microb. Genom. 2016; Shu, Xiao, Yan, & Sun, Front. Microbiol. 2016, where phylogentic trees of both PilA-N and PilA-C were already presented;

None of the previous studies recognized that PilA-N and PilA-C naturally come together to form a filament with very low conductivity. Since 2005, thousands of papers, including some of our own, have claimed that *Geobacter* nanowires are pili made up solely of PilA-N. All previous studies have disregarded the direct role of PilA-C in the formation of *Geobacter* pili and assumed that aromatic residues in PilA-N are closely stacked. Our work is the first to show that *Geobacter* does not form surface-displayed T4P filaments under nanowire-producing conditions. We further show that there is no evidence of any PilA-N-only filament, or conductive structure, even when *Geobacter* pili are observed on the bacterial surface. We show for the first time that PilA-N and PilA-C come together to form a novel heterodimeric structure, which has not reported previously for any T4P or pseudopili. We also show that these novel PilA-N-C filaments are required for secretion of cytochrome nanowires. These results are consistent with a T4P system that assembles periplasmic filaments from a heterodimeric subunit to secrete microbial nanowires, thus having a functional role similar to T2SS pseudopili. We have now further clarified the novelty and significance of these findings in the revised manuscript.

they have also taken the next step and tested the function of the "original" full-lengths PilA-N-C experimentally.

This work (Ref. 47) on an artificial PilA-NC fusion has several issues that limit the degree to which it can be meaningfully compared with the present work on the native PilA-N-C heterodimer filament. First, there is no direct evidence of the claimed PilA-NC fusion filaments. The presence of PilA-NC fusion protein at the monomer level was observed by the authors only at extreme pH 2 conditions. Second, the conditions used by the authors to purify pili (pH 10.5) destroy the native *Geobacter* pili presented in our work, which raises additional doubts that the filaments examined in that study could be composed of a PilA-NC fusion polymer and limit the degree to which the filaments examined in that study can be compared to the *Geobacter* pili we have studied. Finally, the structure and function of an artificial PilA-NC fusion protein could be very different than the native PilA-N-C filament because in the native structure, the C-terminus of PilA-N is not located in such extremely close proximity to the N-terminus of PilA-C. Therefore, we think that this artificial PilA-NC fusion is not relevant to *Geobacter* physiology.

58-60: structural conservation of T4P head domain (including PilA-C) is not that high: the only highly conserved feature is a 3 stranded β-sheet (see also Fig. 3a).

We have further clarified in the revised manuscript that the purpose of these comparisons is to emphasize that PilA-N-C filaments do not show any structural or functional behaviour consistent with typical T4P. We recognize that the functional and structural diversity within T4P means that not every feature common to many T4P will be present in each one. We agree with the reviewer that the absence of any single one of these features is not conclusive, though our combined localization and functional studies, in conjunction with the structure, demonstrate that PilA-N-C pili are unlikely to function as typical T4P filaments. For example, replacing *Geobacter* pili with T4P does not restore the secretion of microbial OmcZ nanowires (Fig. 5c).

Fig. 1B and l. 62-65: phylogenetically, the Geobacter PilA lineage forms a subgroup of T4P, a fact well-hidden in the displayed, unrooted tree (where only bootstrap support for the PilA-N-C branch is given, not for its affiliation with either pseudopilins or T4P).

In the revised manuscript, we have added the bootstrap value for PilA-N-C in Fig. 1b and to each point in Supplementary Fig. 1b. The only conserved branch is the PilA-N-C pili group, while the affiliation to T4P or pseudopili are not consistent. Our phylogenetic analysis thus show that these heterodimeric PilA-N-C pili are unique and lie along the continuum between T4P and pseudopili.

l. 66-69: this hypothesis is not novel or original either, it has already been presented by Wang et al, Cell 2019.

The hypothesis of PilA-N based pseudopili in the discussion of our previous publication (Ref. 2) was presented there without any direct experimental evidence. In this work, we show that there is no evidence of *Geobacter*-produced PilA-N filament, and that extracellular PilA-N-C filaments are displayed on the bacterial surface except in artificial conditions where PilA-N and PilA-C are overexpressed in the absence of cytochrome nanowires. Therefore, we believe that our finding that *Geobacter* pili are non-conductive, primarily periplasmic, and are involved in the secretion of cytochrome nanowires is a fundamental change in understanding of microbial nanowires.

l.76-85: this is not an indication for pseudopili, as PilA of T4P are also known to be anchored to the inner membrane as reservoirs for T4P production, so this result is not surprising; in contrast, the absence of PilA-N in the extracellular fractions is unexpected, as PilA has previously been detected (e.g., Tan et al. 2016; Wang et al., 2019).

The above cited papers, and many other studies of *Geobacter* filaments, contain cellular contamination due to harsh methods used for producing an “extracellular fraction” via shearing filaments using vortexing or blending in a high pH buffer. This includes both of our previous publications mentioned by the reviewer (Ref. 2, 50), as evident by the presence of the periplasmic 50 kDa form of OmcZ in the “extracellular fraction” obtained with the methods used in those papers (Fig. 4b in the manuscript). The present manuscript is the first study that collected *Geobacter* pili directly from the cell-free supernatant without using any shearing method and performed detailed subcellular localization of pili and cytochrome proteins. Mass spectrometric analysis of our samples confirm that they do not contain any cellular contamination (Extended Data. Fig. 1, Supplementary Data Fig. 2). Therefore, we are confident in our finding that PilA-N-C filaments are periplasmic for the wild-type and nanowire-producing conditions and extend outside the cell only under artificial conditions (Fig. 1g, Extended Data Fig. 2). These results are indeed very unexpected because all previous studies assumed that *Geobacter* pili are T4P filaments which by definition should be present on the cell surface.

l. 77 and Fig. 1c, d, e: what is the specificity of the antibodies used, i.e. which epitope do they target? Also, the quality of the Immunoblot images is low, and no size markers are included: it is difficult to discriminate individual lanes, and the densitometric analysis becomes uncertain.

As suggested by the reviewer, the methods have been revised to include the peptide sequence targeted by the antibodies used in this study. We have now included the entire immunoblots for all Main Text and Extended Data Figures in Supplementary Figures. We have now provided additional high-quality immunoblots to eliminate any possible confusion.

l. 80-82: GroEL was included as control for the cytoplasmic fraction. What was used as control for an extracellular protein (to prove that the extracellular fraction was reliably sampled)? Could the extracellular PilA-N-C have been lost because it is easily shed into the medium?

Our extracellular/secreted sample preparations used a direct concentration of cell-free media using a concentrator with a very small molecular weight cut-off (3 kDa). The exact same method was used to prepare extracellular fractions from *ΔomcS/ΔomcZ*, where PilA-N-C filaments were abundant. The same method was used to prepare extracellular fractions from wild type, where PilA-N and PilA-C were not significantly present. Therefore, we are confident that any extracellular PilA-N-C was not lost during the purification. Our filament preparation and whole cell imaging studies are also consistent with these subcellular localization studies (Fig. 1).

l. 99/Methods: which sample(s) was used for cryo-EM? ΔomcS/ΔomcZ strains, or WT with overexpressed PilA-N-C, or both? This is not clear (would it matter?)

All three strains (WT, *ΔomcS* and *ΔomcZ*) produce identical PilA-N-C filaments when the protein is artificially overexpressed (Fig. 1, Extended Data Fig. 3). This is evident from the identical height and power spectra analysis of these filaments produced under conditions that artificially overexpress PilA-N and PilA-C. We have now clarified in the revised manuscript that the *ΔomcS* strain was used to avoid any interference with cytochrome nanowires.

We have also noted that all cryo-EM studies on cytochrome filaments were performed using cells grown under nanowire-producing conditions that required long-distance (> 100 μm) access to electron acceptors (Ref. 2, 5).

Fig. 2f and g could be improved by showing density as mesh or with lower transparency.

We have revised the figures as per reviewer's suggestion.

l. 116: "random coil" is not the correct term, as these loops have a well-defined structures

We have now corrected the term to "loop regions".

l. 123-126: How are these charge reversals explained by the structure? This is not explained in the text, and not inferable from Figs. 2f,g: please extend/include a SI figure.

As suggested, we have now included an explanation with a new figure (Extended Data Fig. 5c-d).

l. 117-129: have any of the "critical interactions" (electrostatic or hydrophobic, e.g. L47:Y43) been experimentally confirmed or are these just assumptions based on the structure? Please clarify.

We have now clarified in the revised manuscript that all the electrostatic interactions have been experimentally confirmed previously for interactions between PilA-N and PilA-C (Ref. 24). The hydrophobic interactions have been experimentally confirmed for other pili (Ref. 32)

l. 132: "A periodic acid-Schiff staining assay suggested this density could be attributed to an O-linked glycosylation (Extended Data Fig. 8b-d)" this is not described in Methods.

We have now included a detailed explanation in Methods.

l. 135-137: stabilization of PilA-N by the binding of PilA-C could also be taken as argument for the proposed chaperone function of PilA-C (designated Spc, short pilin chaperone, in Liu et al. Environm. Microbiol. 2019). Please discuss.

As suggested, we have discussed that while PilA-C may indeed have an additional role as a chaperone, we demonstrate that PilA-C is a critical part of *Geobacter* pili and forms the outer surface of the filament. We further find no evidence of PilA-N filaments. Our data thus refute the hypothesis proposed by Liu et al. that PilA-N alone constitutes the filament and thereby forms a nanowire whereas PilA-C remains in the inner membrane (Ref. 47).

PilA-N-C vs. T4P comparison:

l. 144-150: while I generally agree that the observed PilA-N-C features (hydrophobic stabilization between neighboring subunits; absence of the α-β loop in the globular head; absence of an "essential" disulfide bridge in the D-region) are more similar to T2SS pseudopili than to T4P, there are two concerns: first, these are only indications, as there is no absolute conservation, and none of these features are mutually exclusive for either pseudopili or T4P;

We have further clarified this in the revised manuscript that the purpose of this comparison is to emphasize that PilA-N-C filaments do not show any behaviour consistent with typical T4P functions. These filaments are not expressed on the surface and do not show any involvement in bacterial adhesion, twitching motility or biofilm formation. Furthermore, replacing PilA-N-C with T4P does not restore the secretion fully (Fig. 4c). Our combined localization and functional studies demonstrate that PilA-N-C are unlikely to function as T4P filaments.

second, in the (likely/unlikely? I cannot judge – but please discuss) case that native PilA-N (3 nm) pili exist, the structure of the observed PilA-N-C (6 nm) pili would have a different significance.

As suggested by the reviewer, we have now performed additional experimental analysis that further suggest the lack of any PilA-N-only filament. Our analysis shows that due to a highly hydrophobic surface, filaments solely made up of PilA-N are unstable and energetically unfavourable compared to PilA-N-C filaments (Extended Data Fig. 5b).

We have also discussed in the revised manuscript that all structural studies of filaments by *Geobacter* grown under nanowire-producing conditions failed to find any filament consistent with T4P (Ref. 3-5). PilA-N-C filaments were only found on the bacteria surface when the subunit proteins were artificially overexpressed. We also could not find any literature that have reported direct evidence of PilA-N filaments under native conditions. Without evidence, Filman et al. have claimed that some filaments observed in addition to OmcS nanowires are pili (Ref. 4). We have also observed morphologically similar filaments in our sample preparations. Our analysis shows that these filaments are DNA, not pili, as evidenced by cryo-EM reconstruction of these filaments that yielded helical organization and electron density consistent with DNA. We have now discussed this in the revised manuscript (Extended Data Fig. 8).

l. 150-153: I do not understand the argument that absence of disulfide bridges (which are more common in extracellular proteins) should be indicative of a pseudopilus: pseudopili are located in the periplasm which is not an intracellular compartment with reducing conditions, but rather an oxidizing environment favorable for disulfide bridge formation.

Disulphide bridges are common in T4P and confer high stability critical for several functions (Ref. 32). In contrast, PilA-N-C filaments are less stable than T4P (Extended Data Fig. 7), and can be

easily detached from the cells without any physical shearing typically required to separate T4P (Ref. 34). This observed lower stability is consistent with the lack of disulphide bridges in these filaments. Thus, the purpose of the comparison between T4P and *Geobacter* PilA-N-C pili is to emphasize that PilA-N-C filaments do not show any behaviour consistent with typical T4P filament structure or function. We have further clarified this in the revised manuscript.

lines 154-161: I do not understand the rationale for these functional studies (cell adhesion and twitching motility): the proposed function for PilA in G. sulfurreducens is e- pili/nanowire formation, not adhesion or motility. What are you disproving here?

We have further clarified in the revised manuscript that the purpose of this comparison is to emphasize that PilA-N-C filaments do not show behaviour consistent with any typical T4P filament function such as cell adhesion and motility and thus cannot function as T4P. We also show that these filaments show very low conductivity and cannot function as nanowires.

Fig 3b: V9 appears twice in PilA-N-C: the lower V9 should be labelled as E5

We have corrected this error in the revised manuscript.

Fig 3f: it is unclear for me if the aromatic residues shown here are only from PilA-N (as in the theoretical model, Fig. 3e) or really include those from PilA-C (as indicated in the legend). Please add a closeup with model and density showing the aromatic residues for the combined PilA-N-C to SI Figure S5.

As suggested by the reviewer, we have added these comparisons (Extended Data Fig. 3g-h).

Lack of PilA-N-C nanowire structure & function:

Fig. 3h, i: this figure is virtually identical to Fig. 4f, g, published in Wang et al (Cell 2019), except that only part of the data are shown in Fig. 3h, and Wang 4g show a logarithmic scale: are these really original, new data?

These are entirely original data. The compared conductivity found in Wang et al (*Cell* 2019) is also between individual PilA-N-C filaments and OmcS nanowires. Although the identity of the individual PilA-N-C filaments had not been identified in time for that work, nonetheless, they were prepared from *ΔomcS* strain. In the revised manuscript, we have repeated all the measurements again using identical buffer conditions and have compared the conductivity along the length of individual PilA-N-C filaments with OmcS nanowires.

l. 166-167 and l. 179-181: I am puzzled about this reasoning and result: it is stated that the theoretical prediction for PilA "on the order of mS/cm" was only valid "in the presence of a strong oxidizing agent or a high voltage". But the direct measurement (-0.01 to 0.01 V) showed a conductivity of 1 mS/cm. Isn't that unexpected?

We apologize for the confusion. The theoretical prediction of conductivity of 65-330 mS/cm (Ref. 18) is based upon a hypothetical model of a PilA-N-only filament with aromatic residues within 3.5 Å of each other (Fig. 3e in the manuscript, Ref. 36). However, the cryo-EM structure showed that these residues are in fact > 10 Å apart (Fig. 3f in the manuscript) and the measured conductivity is < 1 mS/cm (Fig. 1i). This ~100-fold lower conductivity is expected due to the significantly larger distance between aromatic residues. This measured conductivity is very low for microbial nanowires because it is 20,000-times lower than OmcZ nanowires (Ref, 5). To avoid any confusion, we have rewritten this section in the revised manuscript by focusing on the large discrepancy between the distances between aromatic residues assumed in the theoretical

calculation (< 3.5 Å) using hypothetical PilA-N model versus that seen in the structure (> 10 Å).

Or do you reckon the prediction would be different for PilA-N-C (would the PilA-C domain be insulating or conductivity-increasing?)? How should a direct measurement of conductance in PilA-N look?

Our observed distances between aromatic side chains for PilA-N are comparable to all previous structures of pili filaments (Ref. 50). Therefore, we do not find any evidence that the presence of PilA-C will affect the structure or conductance of PilA-N (Extended Data Fig. 5a-b and Extended Data Fig. 3 g-h). As the filament solely made up of PilA-N does not exist in any of the nanowire-producing conditions, it is difficult to speculate about its conductance without atomic structure. However, in the revised manuscript, we have also discussed the comparison with synthetic proteins containing π-stacking of aromatic side chains that mimic hypothetical PilA-N filament (Ref. 37). Our conductivity measurements and theoretical studies show very low conductivity (~1 μS/cm) due to π-stacking of aromatic chains.

l. 182-186: The original argument for these observation was that OmcS/OmcZ were necessary for electron transfer to e-pili (reviewed in Lovley & Walker; Front Microbiol. 2019); this is not even mentioned here as alternative explanation?

Our studies show that cells do not produce e-pili under nanowire-producing conditions that require long-distance electron transport. Even under artificial conditions when PilA-N is overexpressed, cells do not produce PilA-N filaments but rather PilA-N-C filaments with very low conductivity due to large gaps between aromatic residues. We have also shown that previous studies on filaments that were thought to be e-pili were either OmcS or OmcZ filaments or DNA.

Previous work by some of us had also discussed the possibility that e-pili transfer electrons to monomeric OmcS and OmcZ, based on the presumption that cytochromes could not polymerize into filaments. However, with the discovery that these cytochromes themselves form nanowires that can directly contact electron acceptors, with observed conductivity sufficient to account for previously measured values (Ref. 11), these hypotheses need to be re-evaluated. We have discussed this in the revised manuscript and have cited a detailed review of the physiological role of nanowires (Ref. 11).

l. 198-203 and Fig. 4a: this part is not novel, as it basically reproduces the knock-out experiments of e.g. Liu et al (ISMEJ 2018);

We had discussed this paper in the original manuscript (Ref. 12). However, none of the previous studies have demonstrated that the deletion of PilA-N inhibits the translocation of OmcS and OmcZ nanowires. The paper referred to by the reviewer does not mention PilA-C, OmcZ or cytochrome nanowires. We have also performed additional experiments that demonstrate a direct role of pili filaments in the translocation of cytochrome nanowires (Extended Data Fig. 9).

there are two interesting points, though: (i) in the present study, OmcS secretion is not completely inhibited by deleting PilA, as judged from the weak band in Fig. 4 (left lower panel);

We have combined biochemical studies with TEM imaging that shows that OmcS nanowires are not displayed on the surface of Δ*pilA*-N cells (Fig. 4b in the manuscript, Extended Data Fig. 9h).

and (ii) when Liu et al (ISMEJ 2018) deleted PilB, the Assembly ATPase, extracellular OmcS (and other cytochrome) levels were unchanged although pilus assembly was inhibited. This is in my view a strong argument against an essential role of PilA-N-C in Omc secretion or more generally in a T2SS.

There is no evidence in Ref. 12 by Liu et al. that pili are assembled into filaments and deletion of PilB disrupts their assembly. All the studies are limited to monomeric proteins. Most importantly, Liu et al. used purification conditions that result in periplasmic contamination, as we have discussed earlier. We have now included direct, side-by-side comparison of the protocol used by Liu et al. which demonstrates that these studies are significantly impacted by periplasmic contamination (Supplementary Fig. 14). Our protocol avoids any such contamination and shows gels in agreement with TEM and AFM images that cells do not display OmcS on their outer surface upon deletion of *pilA*-N (Fig. 4b, Extended Data Fig. 9h).

Furthermore, previous studies in *Pseudomonas aeruginosa* have shown that although deletion of *pilA* inhibits secretion, deletion of *pilB* does not affect the secretion [Lu et al. *Mol. Micro.* 25, 247 (1997)]. This is not unexpected because T4P can be also assembled by T2SS (Cisneros et al. *Mol. Micro.* 86, 805, 2012). Therefore, it is possible for pili to be assembled by T2SS in the absence of PilB and continue the secretion of proteins as observed in these previous studies.

203-219: also in these experiments, OmcS is apparently translocated, although PilA-N-C is replaced by the PilA of Pa or Gu. First, please show these data. How massive is the OmcS translocation?

As suggested, we have discussed that the translocation of OmcS is described detail in our previous publication (Ref. 46). However, replacing *Geobacter* pili with T4P does not restore the secretion of OmcZ nanowires (Fig. 4c). Combined with the observation that *Geobacter* pili are not present on the bacterial surface under nanowire-producing conditions, our studies show that the translocation of cytochrome nanowires is not similar to T4P-based protein secretion (Ref. 49).

Even for OmcZ, there seems to be a slight translocation with the Pa-PilA (Figure 4a). This is not mentioned in the text.

There is no translocation of OmcZ nanowires in Pa-PilA. We have clarified in the revised manuscript that OmcZ exists in two forms – an extracellular, small form (30 kDa) which assembles into nanowires (Ref. 5) and a periplasmic, large form (50 kDa). We have included both forms in Fig. 4 to show that OmcZ is not secreted in the absence of PilA-N-C filament.

Furthermore, previous experiments, involving the last author, have also shown that replacing PilA by Pa-PilA does not impair OmcS translocation (Liu et al., AEM2014). So again, these results are not entirely novel or original, nor do they support the hypothesis "that the unique structure of the PilA-N-C filament is critical for proper translocation of OmcS and OmcZ nanowires".

All previous studies, including that mentioned by the reviewer, have been limited to monomeric OmcS and the existence of PilA-N-C filament and cytochrome nanowires were not known. This is the first study to show that in the absence of PilA-N-C filaments, neither OmcS nanowires nor OmcZ nanowires are translocated extracellularly. The complete suppression of OmcZ nanowire translocation in the absence of PilA-N-C filament in the Pa-PilA strain further highlights that the unique structure of PilA-N-C is required for the translocation of these highly conductive nanowires. The studies with the Pa-PilA strain suggests that the presence of T4P filaments with

somewhat similar morphology to PilA-N-C filaments is sufficient to rescue the translocation of OmcS but not OmcZ. We have further clarified this in the revised manuscript.

Figure 4e, f: in summary, the model presented here is intriguing but still rather hypothetical, and except for the (beautiful) Cryo-EM structure, the current study has contributed very little novel data to prove those hypotheses.

In the revised manuscript, we have performed additional experiments that show direct evidence for the secretory role of PilA-N-C filaments (Extended Data Fig. 9). Specifically, we show that the secretion for both OmcS and OmcZ nanowires could be restored by the in-trans expression of an episomal copy of *pilA-N* and *pilA-C* that reassembles the PilA-N-C filaments. We have also provided insights into the secretion mechanism by discussing the pili-based secretion model in further detail (Fig. 4).

SI Methods:

(unfortunately without line numbers)

We have now added the line numbers.

Phylogeny: were site conservation filters used for the PilA phylogeny? The uncertainty of the alignment will otherwise translate into quite uncertain branchings. Please give bootstrap values for all branches.

As suggested, we have included all the bootstrap values in our phylogenetic tree (Fig. 1b and Supplementary Fig. 1b).

PilA-N-C purification: please add the recipe for Sorensen's buffer

We have now added the recipe in the methods.

MS analysis: were all bands visible on the gel? How much protein was loaded? Did you try to analyze the region where PilA-N-C was expected to migrate, just in case?

To analyse all the proteins in our samples, we have performed solution MS which yielded results consistent with the MS of the gel bands (Supplementary Data Fig. 2 and Ext. Data. Fig. 2).

Subcellular fractionations: how many repetitions were made? What were the controls (antibodies) for intra/extracellular, membrane and periplasmic proteins?

All the information suggested by the reviewer is now provided in the methods section.

SI Extended data:

Figure S1: for the Geobacter PilA alignment, please indicate the end of PilA-N and the start of PilA-C.

As suggested by the reviewer, we have now added a marker to indicate the ending of PilA-N and the starting of PilA-C.

Figures S2, S3, S8: the molecular weights of the PilA preparations seems to differ in the different figures, for PilA-N around 10-12 kDa (instead of 6.6), for PilA-C from 15-25 kDa! Please provide complete images with clear size markers instead of small cut-outs.

As suggested by the reviewer, we have provided complete images with clear size markers (Supplementary Fig. 5-9). The molecular weights for PilA-N and PilA-C were confirmed by mass spectrometry (Extended Data Fig 1).

Table 1: Ramachandran plot – with only 83.85% "Favored", I recommend to use Isolde, Namdinator, or a similar method for improving Ramachandran statistics of the models based on maps in the 3.8/3.9 Å resolution regime.

As suggested by the reviewer, we have applied these methods for improving Ramachandran statistics (Supplementary Fig. 5), and we thank the reviewer for the suggestions.

Reviewer Reports on the First Revision:

Referee #3 (Remarks to the Author):

This manuscript describes the structure of Geobacter pili as polymers of PilA-N and PilA-C heterodimers, shows that these filaments are not conductive, and reveals that their role in electrogenesis is instead to aid in secretion of cytochromes OmcS and OmcZ. These data will resolve a long standing debate in the field about the nature of nanowires, and provide the first example of a heterodimeric subunit whose structure and function appear to be more similar to those of the type II secretion system. The authors have done a good job of revising their initial submission in response to the critiques provided, and I am satisfied with the revisions. Some remaining minor points:

1. The abstract states "Deletion or substitution of pilA-N with T4P of other microorganisms, results in loss of secretion of OmcS/OmcZ nanowires..." but the manuscript shows only OmcZ secretion is impacted when heterologous pilins were supplied.

2. Figure 1a legend does not mention what '97' is. There is a typo in the legend of Figure 4 (showiing).

3. The blot in extended Figure 1a is of poor quality and should be replaced. For both extended and supplementary figures, please indicate in the legends the expected masses of PilA-N, PilA-C, OmcS, OmcZ(s) and OmcZ(L) to aid readers not familiar with Geobacter.

Referee #4 (Remarks to the Author):

This is a thorough revision of a manuscript I had for review previously.
The authors have done a good job to more clearly present their story, the supporting data (including additional experiments), and to discuss their findings in relation to previous literature. As a result, the revised version is much more convincing and focused, and I would like to congratulate the authors to this important study: this will clearly change the view of nanowires and the role of PilA, including a convincing rejection of the "e-pilus" concept.

I have only a few minor points that the authors may want to check prior to publication:
Main text, l. 211-217: the argumentation around the T2SS knock-outs is not clear - why is it remarkable (for PilA function, I assume?) that OMC translocation in G. sulfurreducens is unaffected by deleting its incomplete T2SS when it still has a complete T2SS? And what do you infer from the lack of secretion defects in Shewanella (with classical T2SS) in the absence of pili?

SI/response to reviewers: it looks as if you have been a bit sloppy towards the end and have not executed all changes:
Phylogeny: in the last review, I asked "were site conservation filters used for the PilA phylogeny? The uncertainty of the alignment will otherwise translate into quite uncertain branchings. Please give bootstrap values for all branches." You answered the bootstrap part ("As suggested, we have included all the bootstrap values in our phylogenetic tree (Fig. 1b and Supplementary Fig. 1b") but not the site conservation filter/tree topology part. Please address this.

Subcellular fractionation: I asked "how many repetitions were made? What were the controls (antibodies) for intra/extracellular, membrane and periplasmic proteins?" You replied "All the information suggested by the reviewer is now provided in the methods section." but the text (SI Methods, l.303-307) reads "G. sulfurreducens cells were fractionated using a previously published protocol with following modifications. 300 ml of late-log phase culture grown in NBAF was pelleted at 6,000 x g for 15 minutes at 4 °C. The supernatant was filtered through a 0.22 µm filter and concentrated via a 10 kDa cut off Amicon concentrator. The final concentrate was labelled as extracellular fraction. Other fractions were prepared as described previously." i.e., no trace of the information I asked for.

SI Methods, l. 310: reads "(put a reference here)" - yes, please do so!

Extended Data, Table 1: I suggested methods to improve the Ramachandran statistics of the models. You replied "As suggested by the reviewer, we have applied these methods for improving Ramachandran statistics (Supplementary Fig. 5), and we thank the reviewer for the suggestions." Yet neither the methods are mentioned nor the value has changed. Did you actually apply any f the suggested methods?

Extended Data, Figure 8a: this is a Figure from Filman et al. (2019), which should be clearly stated in the legend.

Author Rebuttals to First Revision:

Point-by-point response to all reviewers' comments. Comments are in bold and revisions to the manuscript are in *italics* here and are marked in red in the revised manuscript.

Reviewer-3:

This manuscript describes the structure of Geobacter pili as polymers of PilA-N and PilA-C heterodimers, shows that these filaments are not conductive, and reveals that their role in electrogenesis is instead to aid in secretion of cytochromes OmcS and OmcZ. These data will resolve a long standing debate in the field about the nature of nanowires, and provide the first example of a heterodimeric subunit whose structure and function appear to be more similar to those of the type II secretion system. The authors have done a good job of revising their initial submission in response to the critiques provided, and I am satisfied with the revisions. Some remaining minor points:

1. The abstract states "Deletion or substitution of pilA-N with T4P of other microorganisms, results in loss of secretion of OmcS/OmcZ nanowires..." but the manuscript shows only OmcZ secretion is impacted when heterologous pilins were supplied. Due to word limit for the abstract, we had to shorten this sentence. We thank the editor for allowing use to extend the abstract to clarify this statement as follows: *Secretion of OmcS and OmcZ nanowires is lost when* pilA-N *is deleted and restored when PilA- N-C filaments are reconstituted. Substitution of* pilA-N *with T4P of other microorganisms also causes loss of secretion of OmcZ nanowires.*

2. Figure 1a legend does not mention what '97' is.

We have now explained that 97 is the bootstrap value of PilA-N-C branch, indicating the conservation of PilA-N-C pili group. All the values for each branch are shown in the Supplementary Figure 2.

There is a typo in the legend of Figure 4 (showiing).
We have corrected this typo**.**

3. The blot in extended Figure 1a is of poor quality and should be replaced.
We have replaced this blot.

For both extended and supplementary figures, please indicate in the legends the expected masses of PilA-N, PilA-C, OmcS, OmcZ(s) and OmcZ(L) to aid readers not familiar with Geobacter.
We have indicated the expected masses in the figure legends.

Referee -4:

This is a thorough revision of a manuscript I had for review previously.

The authors have done a good job to more clearly present their story, the supporting data (including additional experiments), and to discuss their findings in relation to previous literature. As a result, the revised version is much more convincing and focused, and I would like to congratulate the authors to this important study: this will clearly change the view of nanowires and the role of PilA, including a convincing rejection of the "e-pilus" concept.

I have only a few minor points that the authors may want to check prior to publication:
Main text, l. 211-217: the argumentation around the T2SS knock-outs is not clear - why is it remarkable (for PilA function, I assume?) that OMC translocation in G. sulfurreducens is unaffected by deleting its incomplete T2SS when it still has a complete T2SS? And what do you infer from the lack of secretion defects in Shewanella (with classical T2SS) in the absence of pili?
Though both *Geobacter* and *Shewanella* possess complete, classical T2SS and secrete cytochromes extracellularly, the secretion of these cytochromes is affected by deletion of T4P in *Geobacter* only. This suggests that the mechanism of secretion of some or all of these extracellular cytochromes is not equivalent in the two bacteria, thereby highlighting the importance of T4P in the secretion of OmcS/Z in *Geobacter* and challenging what might have been the default assumption – that T2SS is responsible for extracellular cytochrome secretion in *Geobacter* as it is in *Shewanella*.

For the sake of brevity and to address the lack of clarity identified by the reviewer, we have simplified this discussion as follows:

Other species that use classical T2SS for extracellular translocation of c-type cytochromes do not show any secretion defect in the absence of pili. In G. sulfurreducens, deletion of

pilA-N does inhibit the extracellular translocation of OmcS and OmcZ, underscoring the involvement of pili in the secretion of nanowire-forming cytochromes.

SI/response to reviewers: it looks as if you have been a bit sloppy towards the end and have not executed all changes:

Phylogeny: in the last review, I asked "were site conservation filters used for the PilA phylogeny? The uncertainty of the alignment will otherwise translate into quite uncertain branchings. Please give bootstrap values for all branches." You answered the bootstrap part ("As suggested, we have included all the bootstrap values in our phylogenetic tree (Fig. 1b and Supplementary Fig. 1b") but not the site conservation filter/tree topology part.

Please address this.
We have now clarified that the tree topology part is used for the PilA phylogeny. Replotting the tree as a rooted tree (shown in Supplementary Fig. 1b) also showed that PilA-NC is clustered into a separate group with high conservation (Supplementary Fig. 1c).

Subcellular fractionation: I asked "how many repetitions were made? What were the controls (antibodies) for intra/extracellular, membrane and periplasmic proteins?" You replied "All the information suggested by the reviewer is now provided in the methods section." but the text (SI Methods, l.303-307) reads "G. sulfurreducens cells were fractionated using a previously published protocol with following modifications. 300 ml of late-log phase culture grown in NBAF was pelleted at 6,000 x g for 15 minutes at 4 °C. The supernatant was filtered through a 0.22 μm filter and concentrated via a 10 kDa cut off Amicon concentrator. The final concentrate was labelled as extracellular fraction. Other fractions were prepared as described previously." i.e., no trace of the information I asked for.
We have now clarified that at least three biological replicates were used for analysis and all the controls have been discussed in the manuscript. For example, the GroEL was used as the control for cellular protein and confirmed no cross-contamination between periplasmic and cytoplasmic fractions (Supplementary Data Fig. 3).

SI Methods, l. 310: reads "(put a reference here)" - yes, please do so!
We have included the reference.

Extended Data, Table 1: I suggested methods to improve the Ramachandran statistics of the models. You replied "As suggested by the reviewer, we have applied these methods for improving Ramachandran statistics (Supplementary Fig. 5), and we thank the reviewer for the suggestions." Yet neither the methods are mentioned nor the value has changed. Did you actually apply any of the suggested methods?
We have now clarified that the values were not improved after applying the suggested Namdinator remodeling method (Supplementary Table 1) and showed improvement using ISOLDE (Supplementary Table 2).

Extended Data, Figure 8a: this is a Figure from Filman et al. (2019), which should

be clearly stated in the legend.
We have included this reference in the figure legend.