Responses to Anonymous Referee #1

We thank the reviewer for his/her constructive comments that we address below line by line (responses are in italic, text additions to the revised manuscript are in blue).

This manuscript describes observations of high temperature ice nucleation activity of some common proteins and a virus. The introduction and discussion point toward their relevance as potential cloud aerosols. There is, however, very little background on the properties of proteins/compounds that serve as ice nucleators. The efficacy of an aerosol to serve as an INP largely depends on its chemical/mineral makeup, morphology, and size. None of these properties are introduced and sufficiently discussed.

We added a paragraph to the revised manuscript discussing surface properties that are considered to promote ice nucleation (Page 3, lines 13 – 25):

Heterogeneous ice nucleation is considered to arise from the ability of surfaces to order water molecules in an ice-like pattern. The arrangement of water molecules at a surface depends on surface charge and functional groups (Glatz and Sarupria, 2016; Abdelmonem et al., 2017; Pummer et al., 2015). A relevant role is attributed to surface OH and NH groups that are able to form hydrogen bonds to water molecules. Their number and arrangement have been used to explain IN activity of different mineral surfaces (Pedevilla et al., 2007; Hu and Michaelides, 2007; Glatz and Sarupria, 2018; Kumar et al., 2019b). A lattice match between ice and the ice-nucleating agent is often considered a prerequisite for heterogeneous ice nucleation. Yet, while some IN active substances such as AgI (Marcolli et al., 2016) and 2D-crystalline films formed by long-chain alcohols (Popovitz-Biro et al., 1994; Zobrist et al., 2007; Qiu et al., 2017) exhibit a lattice match, others such as quartz (Kumar et al., 2019a) do not, and even others such as BaF_2 exhibit a lattice match but fail to be IN active (Conrad et al., 2005). The difficulty to pinpoint surface properties that are required for heterogeneous ice nucleation may be explained by growing evidence that it is not the whole surface that is able to nucleate ice but just special nucleation sites (Vali, 2014; Vali et al., 2015), which may arise through defects or impurities. Applying classical nucleation theory to heterogeneous ice nucleation yields nucleation site areas in the range of 10 – 50 nm^2 required to host an ice embryo of critical size (Kaufmann et al., 2017).

In addition, the rationale for the experimental design and selection of these specific proteins for analysis is not presented.

The selected proteins cover a broad variety of forms, sizes and functions as outlined in Sect. 2.1. The rationale was to elucidate whether proteinaceous material has an inherent ability to nucleate ice, irrespective of its function. We add a sentence explaining the rationale of this study (page 3, lines 31 – 32):

So far, investigations have been focused on proteins that are expressed by organisms to nucleate ice. Here we examine whether proteins as a type of macromolecules have an inherent ability to nucleate ice.

I believe the results presented are interesting in that they provide insight into the (unexpected) types of proteinaceous compounds that can serve as efficient INPs. However, there are a number of items in the presentation that must be clarified before this article should be considered for publication.

Specific comments:

Pg. 2, Lines 23-25: The first two sentences give the impression of a discussion on marine INPs but are unrelated to the content that follows in this paragraph. Please considering editing for clarity.

These three lines are a paragraph on their own. This paragraph and the previous one discuss sources of biological aerosols in terms of areas (terrestrial and marine). The next paragraph treats the nature of biological material that has been found to nucleate ice.

Pg. 2, Line 30 and throughout manuscript: Please do not capitalized the species name, i.e, Pseudomonas syringae.

Thanks for pointing this out. We have corrected to "syringae" throughout the manuscript.

Pg. 2, Line 33: Suggest replacing "populate surfaces" with "leaf surfaces" or describing the bacteria as epiphytes.

We specify "leaf surfaces" in the revised manuscript.

Pg. 3, Line 4: "Here we focus on proteins, which are the most or second-most important biological INPs." Please explain the basis for this statement. Presumably it is in reference to an atmospheric context.

In response to a comment of referee #2, we replaced this sentence by:

So far, investigations have been focused on proteins that are expressed by organisms to nucleate ice. Here we examine whether proteins as a type of macromolecules have an inherent ability to nucleate ice.

Pg. 5, Line 35: Please explain what this means (skimming David et al. 2019 didn't help easily figure this out).

We now mention the purpose of the bath leveler in the text:

…the bath leveler, which keeps the ethanol bath level constant during a cooling ramp, was used as described in David et al. (2019).

Pg. 6, Lines 30-31: This step would create water loss due to evaporation during the treatment. Was the protein concentration determined after the treatment or was concentration corrected for the evaporated volume?

For the heat treatments, the bottles were loosely closed by a cap so that water loss was low (less than 2 %). We did not correct this water loss, as it was low and we did not exactly quantify it. For clarification, we add to the revised manuscript:

To prevent water loss, the bottles were loosely covered by a cap.

Pg. 7, Lines 21-23: Please explain the rationale for the protein concentrations used in the experiments. For example, why were all not tested at the same molar concentration?

For heterogeneous ice nucleation, the surface area is considered relevant. When the surface area is not determined or not well defined, mass concentration is usually used. Plotting active site densities per mass is a common way to normalize IN activity of biological material (see e.g. Kanji et al., 2017, Fig. 1-5 or Pummer et al., 2015). Therefore, we used for all proteins the same mass concentration in the screening experiment. For the virus, we had to use a lower concentration as it was provided to us at this concentration.

Pg. 8, Lines 12-13: Please explain the basis of this conclusion. Is there evidence from other measurements that the quaternary structure of the protein was disrupted in batch 2?

In both batches, the main component is the correctly folded and assembled apoferritin or ferritin. Yet, there is a tiny fraction of misfolded apoferritin/ferritin that is most probably involved in the formation of larger aggregates. We show later in the manuscript that we were able to confirm the presence of aggregates by DLS.

Pg. 8, Lines 19-21: Unclear how the presence/absence of Fe is inferred. Would dialysis be expected to remove Fe bound to the ferritin protein?

Dialysis is not expected to remove Fe bound to the ferritin protein. We improve the sentence to make clear that we exclude an effect of Fe that is bound to ferritin:

Overall, the IN activity of ferritin is lower than the one of apoferritin, which makes it unlikely that iron plays an active part in ice nucleation by ferritin.

Pg. 13, Lines 13-14: First, the IN protein is in the outer membrane of these bacteria, not their cell membrane. Second, I'm not certain this comparison is a good one since heat-treated IN proteins of P. syringae lose their activity.

We refer only to the outermost membrane, which is now obvious in the text. In addition, please note that these lines do not refer to heat treatment.

Pg. 14, Lines 13-14: The statement that cells must be disrupted to display IN activity is not accurate (e.g., Christner et al. 2008, 319:1214).

We were thinking here of ferritin and other proteins that are not located on the outer membrane of organisms and will not be in contact with the environment when the organism is intact. However, the reviewer is correct that this does not need to be the case in general. Therefore, we removed this sentence in the revised manuscript.

Pg. 14, Lines 22-23: I had trouble following this argument. Are you referring to aggregation in the wet phase? If not, how do dry aerosols become diluted?

We are referring here to dilution during cloud droplet activation and concentration in the aerosol particles.

Figure 2: What are the dotted lines co-plotted? Confidence intervals?

In all frozen-fraction plots, the dotted lines are the results of individual freezing runs. In the revised manuscript, we have added this information now also to Fig. 2.