Atmos. Chem. Phys. Discuss., 12, C12560–C12575, 2013
www.atmos-chem-phys-discuss.net/12/C12560/2013/

Atmospheric Chemistry and Physics Discussions

Interactive comment on "Immersion freezing of ice nucleating active protein complexes" *by* S. Hartmann et al.

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Received and published: 12 February 2013

The authors thank Cindy Morris for her helpful comments and suggestions. Considering the referee's opinions we restructured and rewrote more or less the entire manuscript. Especially the "introduction" and "summary and conclusion" sections have been significantly modified. This makes it sometimes impossible to relate specific referee comments to specific changes in the text. We apologize for the resulting more general references to certain sections of the manuscript.

In the following the referee's comments are marked in italic and the corresponding answer in regular letters.

MAJOR COMMENTS Referee # 2:

The comments below were written before I looked at the comments of the other reviewer who posted his/her remark before mine. Hence they are completely independent of any influence that the previous review could have had on my opinion.

In this work the authors used the LACIS system to evaluate the immersion freezing behavior of the bacterium P. syringae in the form of the Snomax product. Compared to the immersion freezing test of larger droplets of more dense suspensions of Snomax, this is a relatively complicated technique (from the point of view of microbiologists who are well acquainted with the droplet test). However, I do not see the added value of the technique because the conclusions are essentially the same as those gained from the simpler technique in terms of the rates of efficiency of this INA bacterium.

In Murray et al. (2012) it is stated that "to date, relatively few experiments have examined the [ice] activity of bacteria using techniques validated to be free from potential interference such as nucleation by substrates". Most of the previous investigations concerning bacterial ice nucleation were carried out following Vali (1971), thus interference by the substrate cannot be ruled out. Furthermore, previous investigations usually considered a large number of bacteria or bacteria fragments per droplet. As pointed out in Niedermeier et al. (2011), this may affect both, the onset temperature at which ice nucleation can be observed, and the apparent temperature dependence of the ice fraction and related and or derived properties. Another issue are statistical uncertainties when applying the procedure according to Vali (1971), as usually only a small number of droplets is considered. Altogether, despite being widely used, measurements according to Vali (1971) may be affected by uncertainties and/or artifacts. Therefore, we found it necessary to use an admittedly more complicated, however, also more powerful technique for investigating bacterial ice nucleation behavior. Our technique is free of potential interferences from substrates, allows for the considera-

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tion of a statically relevant number of droplets which contain only a small number of bacteria and/or bacteria fragments, allows for particle size segregated measurements and therefore makes possible a more detailed investigation of bacterial ice nucleation behavior. Finally, we take a different perspective in quantifying the bacteria's ice nucleation behavior. First, we are not looking at the number of bacteria, but the number of ice nucleation active protein complexes being present in the investigated droplets and second we use ice nucleation rates instead of onset temperatures and frozen fractions. We consider this the more physically based approach, as there is strong evidence that heterogeneous ice nucleation is fundamentally a stochastic process (e.g., Niedermeier et al., 2011). In this context it should be noted that e.g., Yankofsky et al. (1981) and Brinkmann (2010) in their investigation found a time dependence (short time scale) of the ice nucleation process which is indicative for the stochastic nature of the ice nucleation process in their experiments and hence supports our approach.

Altogether, with this manuscript we

- provide means to investigate the ice nucleation behaviour of ice nucleating protein complexes attached to intact P. syringae bacteria cells and cell fragments,
- provide temperature dependent ice fractions and for the first time a nucleation rate for a single ice nucleating protein complex,
- found similar temperature dependencies as e.g. Maki et al. (1974), Yankofsky et al. (1981), Lindow et al. (1982) and Wood et al. (2002) and therewith confirmed both, previous results and the drop freezing method introduced by Vali (1971),
- showed that the determined ice nucleation rate is valid for both protein complexes attached to intact cells as well as cell fragments.

So we are convinced, that the present manuscript offers a significant amount of original and new material.

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The manuscript was restructured and rewritten. Special focus was hereby given to the "introduction" and "summary and conclusion" sections, which now in our view contain a significantly improved motivation, definition of the considered INA protein complexes, broader view of the existing literature, and some suggestions concerning the implications of the gained results for atmospheric ice nucleation and its description in numerical models.

The authors claim that they are working with a small number of protein complexes per drop and that they can evaluate the number of protein complexes in each drop. One of the assumptions that they make for these calculations is that the absence of freezing means the absence of the protein complex. However, this has not been verified.

We are interested in the ice nucleation behavior of ice nucleation active protein complexes, so why should we care about protein complexes, which don't even nucleate ice at temperatures even below $-20\,^{\circ}$C? With the improved definition of INA protein complex and the modified description of the experimental results (section 3.1) we hope to avoid further misunderstandings.

Furthermore, individual protein complexes are not dispersed in the Snomax product. This product consists of whole bacterial cells that each harbor numerous protein complexes on their surfaces. It is possible to disperse the cells but I do not understand how the protein complexes were dispersed without breaking the cells and thus damaging the ice nucleation efficiency.

According to Govindarajan and Lindow (1988) and Lindow et al. (1989) cell fragments with attached protein complexes are the smallest ice nucleating unit to be expected as pieces of cell membrane are required to keep the complex together. The presence

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of cell fragments is most likely due to the freeze drying during production and/or the atomization technique applied in the course of our experiments. It should be noted, that even if we destroy some of the INA protein complexes during particle generation, it would not change the nucleation rate given in this paper, because for its determination we only need to know the number of ice nucleation active protein complexes being present in the droplets which can easily be determined from the measurements.

Furthermore, in our view, there is a clear contradiction between the statement of the referee that bacterial cells harbor numerous protein complexes on their surfaces, and what we were able to find in the literature. E.g. Orser et al. (1985), Southworth et al. (1988), Wolber et al. (1986), Lindow et al. (1989), Schmid et al. (1997) and Attard et al. (2012) claim that the majority of bacteria cells have none, one, or a little more than one ice forming sites on their surface. This is in line with our findings, that, considering particles in the size range around 800 nm, where we expect to find the intact *P. syringae* cells, only every second particle contained an INA protein complex. Especially the introduction and section 2.1 have been rewritten to more clearly describe and discuss our views and approach.

It seems as if it would be more accurate to state that they can calculate the number of ice nuclei per drop.

We would prefer to stick to our nomenclature as in our view one INA protein complex in a droplet is enough to induce freezing. However, we included a better definition of the complex into the manuscript as already mentioned above.

In this case, it is not clear what new information about ice nucleation activity is provided with this technique.

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Please, see above.

The CHESS model could be useful for predicting the INA at a given temperature from data at other temperatures and hence could be used to parameterize models where the abundance of biological ice nuclei in the atmosphere is a parameter. However, they have not demonstrated the real predictive validity of this model for bacteria.

We think we did. Successfully describing the ice nucleation behavior of both INA protein complexes (e.g., Wood et al. (2002) and our data for Snomax^{TM}) and the behavior of *P. syringae* bacteria in different experiments (Maki et al. (1974), Yankofsky et al. (1981) and Lindow et al. (1982)), with the average number of INA protein complexes present in the droplets λ being the only free model parameter, gives a strong hint that the determined nucleation rate and the CHESS model are valid for bacteria. However it should be noted, that for e.g. atmospheric applications, λ has to be known. Assuming each bacteria to have one INA protein complex, λ corresponds to the average number of *P. syringae* bacteria per droplet. If only a certain fraction of bacteria features an INA protein complex, λ decreases accordingly. In other words, the presented nucleation rate, together with a proper value of λ, are feasible to be used in models, in which the abundance of biological ice nuclei in the atmosphere is a parameter.

The "introduction", and especially the "summary and conclusion" section, have been rewritten accounting for the referee's concern. The latter now includes a brief outline how the model could be applied.

p 21323 l 16, This citation, Ward and DeMott, 1989, is not pertinent for the remark about the structure of the protein. These authors did not analyze protein structure.

This citation has been removed from the text.

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p 21324, l 4: "since the seventies of the last century", Change to "since the 1970s

Changed in the manuscript as requested.

p 21324, l 14: "bacteria related particles", Change to "bacterial particles"

Changed in the manuscript as requested.

p 21324, l 14-20: The authors state that it is difficult to quantify and interpret the INA properties of polydisperse INA distributions. Why is this so? This statement should be justified.

This statement was misleading its specific context. However, performing polydisperse measurements generally implies loss of information and might lead to misinterpretations. This is due to the fact that polydisperse measurements usually don't allow for the separation of, size, composition and mixture (external vs. internal) effects. E.g. Möhler et al. (2008) assumed in their measurements concerning the ice nucleation behavior of particles generated from a Snomax ^{TM} solution/suspension, that only particles with sizes around 800 nm were inducing ice nucleation. This led to far too low ice fractions being reported. Only by performing monodisperse measurements we were able to figure out that the average number of INA protein complexes per particle generated from a Snomax^{TM} solution/suspension correlated with particle volume, and could prove that the determined nucleation rates were no function of particle size. These two pieces of information lead to the conclusion, that we were really quantifying the ice nucleation behavior of single protein complexes. However, we agree with the reviewer,

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that we could have retrieved similar information already from the existing data. But we would have had no proof that the assumptions made were applicable and the numbers retrieved were correct.

Especially the "introduction" has been rewritten to explain needs for monodisperse experiments more clearly.

p 21324, l 20-23: When the authors mention ice nucleation behavior what are the parameters involved? They seem to indicate that threshold temperature is not part of nucleation behavior. It would be useful if the word behavior could be substituted with something more precise. Overall the authors seem to be saying that there are parameters of nucleation induced by INA proteins that have not yet been characterized (nucleation rate, for example). This paragraph belabors the point; they should just state that and explain why these parameters are important or more informative than threshold temperature.

Quantification of ice nucleation behavior in the framework of this paper implies measurement of temperature dependent ice fractions and the determination of ice nucleation rates as function of temperature. Indeed, we don't consider threshold temperatures the proper parameter in the context of our investigations, as it depends on both, the actual nucleation rate and the number of INA protein complexes being present in the droplets, with the latter being experiment specific and consequently not a general property.

Again, we refer to the significantly modified "introduction" and "summary and conclusion" sections.

p 21326, l 3: consists of deadened, change to consists of non viable

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Changed throughout the manuscript as requested.

p 21356, l 6-7: The Pseudomonas syringae bacteria used in Snomax production are grown, change to The strain of P. syringae used in Snomax production is grown

The whole section has been rewritten.

Materials and Methods

p 21326, section on Snomax. The authors do not explain how the particles of different size ranges are generated and separated.

The whole section has been restructured and rewritten.

Results

p 21330, l 1-14: The authors argue that A and B class INA proteins are not present in detectable amounts among the 1000 to 10000 droplets tested. However, Figure 3 shows are small fraction of ice forming at temperatures warmer than -5C and just a bit colder than -5C. The rates are about 1/1000, so that would mean between 1 and 10 frozen droplets, assuming on average 1 particle per droplet. Why do the authors say that the class A and B nuclei are not detectable? Perhaps it would be better to say that they represent a small fraction of the total nuclei. I would like to remind the authors that this is the typical situation in INA bacteria, that the class C proteins are generally dominant.

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The entire section 3.1 has been significantly modified. This issue is now discussed in more detail, based on the number of ice particles counted, and the shape of the ice fraction curves.

p 21330, l 15-21: The increase of ice fractions with increasing particle size is not surprising. Furthermore, the fact that this increase is not linear with increasing particle size also seems to be intuitive. The protein that is responsible for INA of bacteria constitutes a small fraction of the total surface proteins of this bacterium. The cell size of P. syringae is about 1 μm. Furthermore, the cell membrane holds the INA protein structure to ensure its activity. Hence, very small particles are likely to not have any protein on them. Furthermore, if the particles represent cells that died and lysed during the industrial preparation of Snomax, then the protein configuration necessary for ice nucleation activity might have been lost. These particles would be expected to have lower activity or no activity relative to particles that arise from cells that fraction after lyophilization of the Snomax product. The authors could mention this either here or in the discussion to illustrate that their results are logical.

We now do so, e.g. in the section 3.2.

p 21330, l 24: The authors state that they can determine the number of protein complexes in a droplet. Do they, in fact, mean the number of ice nuclei? Do they assume that each protein complex leads to the formation of only 1 ice nucleus and that all protein complexes freeze under the temperatures tested here? Above, (p 21329, l 21-22) they indicate that the fact that droplets are unfrozen means that they do not contain even a single INA protein complex. But how do they know that? It is possible that certain INA protein complexes are formed in such a way that they are not efficient ice nucleators. I think that it would be advisable for the authors to modify their vocabulary to reflect what in fact they actually measured ice nuclei. This

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remark means that the authors might not really know if they have monodisperse or polydisperse suspensions. If they are referring to particles, then yes they can verify that they have monodisperse suspensions, i.e. that the suspension contains single particles and of a single size. But if they are targeting monodisperse suspensions in terms of INA protein complexes, they do not know that this is true. They only know that they have single ice nuclei on average in a drop, and most of them active at about the same temperature (sharp increase in number of frozen drops and then saturation).

The referee is correct in stating, that we cannot be sure that we are considering monodisperse protein complexes. Nevertheless, we would prefer to stick with the old nomenclature, however, we included a better description of the INA protein complex into the paper. Furthermore, we also mention the implications of differently sized or structured INA protein complexes being present.

A special paragraph concerning this issue has been added to the manuscript.

p 21335, l 17-19: The authors conclude that the INA protein complexes from P. syringae are the most active IN known. This information is surely not new and is well documented in the literature. The authors should indicate what new information they provide via the CHESS calculation that gives new insight into the nucleation capacity of P. syringae. The application of the CHESS model to other systems is illustrated in FIGURE 8. The curves in this figure for the Maki et al data for P. syringae extrapolate based on a single data point. In one case this leads to predicting that there is ice nucleation activity above 0C. There are many more data available in the literature on the INA of P. syringae that the authors could have chosen. Nevertheless, the model predicts what has already been observed experimentally for the ice nucleation spectra due to the protein of P. syringae as best illustrated by Orser et al, Figure 4, pg 363 (Orser, C., et al.: Cloning and expression of bacterial ice nucleation genes in

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Escherichia coli., J. Bacteriol., 184, 359-366., 1985.)

Of course an extrapolation to temperature values higher than $0\,^{\circ}$C is physically nonsensical. As a consequence, the curves are only given up to $-1\,^{\circ}$C in Fig. 8.

Considering the other remarks given by Cindy Morris, there are indeed many publications in which ice nucleation by *P. syringae* was examined. Most of them used the drop freezing method as described in Vali (1971) and show cumulative ice nuclei concentrations per bacteria cell over a range of temperatures. Following Vali (1971), these can be transformed into an ice fraction (f_{ice}), if the number of bacterial cells per droplet or the number of bacterial cells per volume of the solution together with the size of the examined droplets and the respective dilution state is known. However, most of these publications, including e.g. Orser et al. (1985), Wolber et al. (1986) and Lindow et al. (1989) do not report these values, and hence we could not include them in our comparison.

Lindow et al. (1982) did report frozen fractions, so two of their datasets were additionally included in our comparison. However, the dataset with the lowest concentration was not used. The reason being, that there are contradictions in the paper around that particular dataset. Based on a CFU/ml of 3500 and a drop size of 30 microliter, as reported in the paper, one drop has, on average, 105 cells immersed. Also given in the paper is, that 1 out of 50 cells was ice active, which, on average, implies that on average 2 cells per drop should have been ice active. This, in turn, using a Poisson distribution, implies that more than $85\,\%$ of all droplets should have frozen, which was not the case. Therefore, we decided to not consider this data in our comparison.

Fig. 8, its description and the related discussion have been re-written (see section 3.4).

Additionally, throughout the manuscript and particularly in sections 3.4. and 4, the

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novelties of our study are now stated more clearly.

Finally, the authors would like to make brief comment concerning application of different terminology and different interpretation methods in this and earlier studies as mentioned above. The often applied method introduced by Vali (1971) is based on the singular hypothesis and in this study we followed the stochastic hypothesis of heterogeneous ice nucleation. This might lead to different interpretations of similar results.

There are some English usage and punctuation errors. The authors should have the manuscript read by a physicist who uses English as a native language.

We hope most of the errors are eliminated in the revised version of the manuscript.

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