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Authors: Rusconi B, Lienard J, Aeby S, Croxatto A, Bertelli C, Greub G
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Crescent and Star Shapes of Members of the Chlamydiales Order: Impact of Fixative Methods

--Manuscript Draft--

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Corresponding Author:	Gilbert Greub, MD PhD University of Lausanne Lausanne, SWITZERLAND
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	University of Lausanne
Corresponding Author's Secondary Institution:	
First Author:	Brigida Rusconi
First Author Secondary Information:	
Order of Authors:	Brigida Rusconi
	Julia Lienard
	Sebastien Aeby
	Antony Croxatto
	Claire Bertelli
	Gilbert Greub, MD PhD
Order of Authors Secondary Information:	
Abstract:	Members of the Chlamydiales order all share a biphasic lifecycle alternating between small infectious particles, the elementary bodies (EBs) and larger intracellular forms able to replicate, the reticulate bodies (RBs). Whereas the classical Chlamydia usually harbours round-shaped EBs, some members of the Chlamydia-related families display crescent and star-shaped morphologies by electron microscopy. To determine the impact of fixative methods on the shape of the bacterial cells, different buffer and fixative combinations were tested on purified EBs of Criblamydia sequanensis, Estrella lausannensis, Parachlamydia acanthamoebae, and Waddlia chondrophila. A linear discriminant analysis was performed on particle metrics extracted from electron microscopy images to recognize crescent, round, star and intermediary forms. Depending on the buffer and fixatives used, a mixture of alternative shapes were observed in varying proportions with stars and crescents being more frequent in C. sequanensis and P. acanthamoebae, respectively. No tested buffer and chemical fixative preserved ideally the round shape of a majority of bacteria and other methods such as deep-freezing and cryofixation should be applied. Although crescent and star shapes could represent a fixation artifact, they certainly point towards a diverse composition and organization of membrane proteins or intracellular structures rather than being a distinct developmental stage.

*Response to reviewers' comments
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Reviewer #1: This manuscript reports on the analysis of the ultrastructure of four chlamydial species determined by transmission electron microscopy. Depending on the buffer and fixatives used, the bacteria showed different cell shapes ranging from star-shaped to round. The authors concluded that morphologies described as distinct stages in the developmental cycle of these bacteria may represent artifacts caused by fixation.

This manuscript is interesting and not without merit, yet in its present form the data does not support the conclusions, and there are some major issues that need to be addressed.

Determination and quantification of cell shapes was done using image analysis software and statistical methods. Although this is the key step in the analysis the method has not been adequately evaluated and is only very superficially described so that it is impossible to understand what has been done. Specific concerns are:

(i) Classification of cell shapes is based on objects recognized by ImageJ. Have the authors tested how well object recognition works? This is likely highly sensitive to higher cell densities, i.e. whether objects are very close to one another or not. As all the downstream analysis depends on this initial step, it is important to validate this step and to explain how different cell densities were accounted for.

Indeed if the bacteria are in too high number the shapes cannot be recognized anymore since the outline will include more than one bacterium. If the cell density was very high, we would observe a strong decrease in the number of particles analyzed per image. The maximum area included in our analysis was defined as 0.15 pixel^2 as described in the material and method section. Thus, aggregates of many bacteria would fall outside the allowed range. Conditions exhibiting few particles per picture were visually checked but few particles could be seen rather than bacterial aggregates.

Moreover, if the cell density was slightly too high, we would expect a strong increase of the undetermined large particles. We specifically designed this group to recognize aggregates of two to three bacteria. To answer the reviewer's remark, we determined the mean area of particles for each condition without maximum cutoff (Supplementary figure 2). However, we could not observe any correlation between the mean particle area and initial bacterial concentration or the percentage of undetermined large particles.

Thus, in our case we did not encounter the problem of samples with too high cell density, but indeed this method cannot be applied to samples that are too dense. For clarity the following sentence was added

"To rule out that aggregation of bacteria significantly leads to a reduced number of particles counted, we quantified the mean particle area for each condition (supplementary Fig. S2). For none of the bacteria, we could observe a correlation between the mean area and the initial bacterial concentration. However, for *W. chondrophila* and *C. sequanensis* the area was significantly higher for 100mM PBS in 1% osmium tetroxide compared to all other investigated conditions (Fig. S2)."

(ii) Performance of LDA was apparently tested using data from both training and validation sets. However, sensitivity and specificity are provided only for a random data set including the data from the training set. While the performance of the method is interesting if the training set was used, it is crucial to evaluate the performance with data *excluding* the training set. This needs to be added.

The calculation of the method performance has already been evaluated on distinctive training and testing set that are mutually exclusive, as asked by the reviewer. Then, to assess the performance of the LDA on any set selected among the 488 particles, we selected randomly 100 times a training set and the complementary test set. Each of the 100 complementary and mutually exclusive training-test sets was used to train a LDA and to assess the sensitivity and the specificity.To clarify the text in the method section, we modified the following sentences: "Each particle was assigned randomly to a training set (200 particles) or to a test set (288 particles), implying that training and test sets are non-redundant. A linear discriminant analysis (LDA) was trained on the training set. Then, the LDA was used to predict the shape of the remaining 288 manually selected particles. Finally, pictures of each condition were automatically processed through ImageJ and LDA analysis respectively to evaluate the proportion of each shape.

To assess the global performance of LDA on varying training sets, 100 random training sets and each of the complementary test set were used to train 100 LDA and determine in each case the specificity and sensitivity of shape assignment. Analyses were performed using R (RCoreTeam 2012) and package MASS (Venables and Ripley 2002)."

(iii) It is not clear how sensitivity and specificity were calculated. In fact, it seems impossible that the specificity for all classes is close to 100% (Figure 1d) with the sensitivity of e.g. the class "crescent" being only around 75%. If there wasn't another class that is not shown here, the crescent shaped cells should have been classified in one of the other classes, which should result in a decreased specificity.
To better understand the performance and evaluation of LDA, the confusion table should be provided.

The sensitivity was calculated as the ratio between the number of true positives and the number of positives (trues positives + false negatives) for each shape. Similarly, the specificity was calculated as the ratio between the number of true negatives and the number of negatives (true negatives + false positives). As asked by the reviewer, we now provide the confusion table of the LDA used to analyze all images for each particle shape as Table 2.

Shape	True positive	False positive	True negative	False negative	Sensitivity	Specificity
Crescent	35	9	235	9	79.5	96.3
Round	51	1	234	2	96.2	99.6
Convex polygon	57	5	226	0	100	97.8
Star	54	8	223	3	94.7	96.5
Undetermined large	34	3	244	7	82.9	98.8
Undetermined small	28	3	249	8	77.8	98.8

(iv) Relating to the last point, could all recognized objects be assigned to either one of the classes? If not this might have a substantial effect on the quantification of cell shapes.

During the LDA analysis, all objects recognized as a valid particle by ImageJ were classified in either one of the classes. We now specify this point in the text. Moreover, as already mentioned in the text, only particles outside the following criteria were excluded by ImageJ: Circularity: 0.2-1; size: 0.02-0.15 pixel^2.

The chapter on the analysis of cell shapes within cells is very weak. Different cells (Vero cells, amoeba) were used for the infection experiments, and different time points post infection were analysed. Yet, the presence or absence of certain bacterial cell shapes might be dependent on the host cell and is certainly dependent on the progression of the developmental cycle. The obtained images can thus not be compared. This part of the manuscript should either be supplemented with additional data or omitted completely.

Upon suggestion of the reviewer the section on chlamydial shapes within infected cells was removed from the manuscript.

Other points:

The authors seem to assume that the round shape is the natural shape of all bacterial species investigated here, yet they fail to provide any evidence for this. A hypotonic buffer might cause round cell shapes by artificial inflation of otherwise star-shaped cells. This possibility should be considered and respective statements should be qualified. Also, what does storage and freezing at -80°C prior to fixation do to the bacteria? Couldn't this induce additional artifacts?

The round shape is assumed to be the natural stage, as shown in previous studies on *Chlamydiaceae* by freeze deep-etching and freeze-fracture. This information has been added to the introduction :" These round shapes were confirmed by freeze deep-etching (Matsumoto et al. 1976)."

The production of *Chlamydiales* requires the infection of a large amount of cells for a quite low yield. Moreover, to reach a high concentration of infected cells the bacteria must be expanded for up to three weeks. Usually we produce a stock of bacteria in a large amount and then store them frozen in 10% glycerol or SPG for further use. For these reasons we used frozen bacteria and not bacteria from freshly infected amoebae. Also the purification of these bacteria requires ultracentrifugation over a gradient. We cannot exclude that the bacterial shape was not affected by the freezing, but the bacteria are washed and "regenerated" in buffer prior to fixation. This information has been added to the material and method section.

In reference to the reviewer remarks on the possible damage by storage at -80°C we added the following statement in the discussion:" Bacteria were stored frozen at -80°C with 10% glycerol or in

SPG prior to fixation. Although we cannot exclude that this step might influence the morphology of the bacteria, the bacteria are viable and infectious."

Osmolarity is discussed at several occasions as possible cause of artifacts, yet osmolarity was not measured in any of the experiments. This data should be added.

The osmolarity values were added in a supplementary Table S1 in the manuscript.

Additional data on the performance of the purification protocol to enrich EBs should be provided. Different cell shapes might be the result of different proportions of RBs and transitional stages in the EB preparation of different species.

When amoebae are full of *Chlamydia*-related bacteria, these latter differentiate from RBs to EBs and trigger the lysis of the host cell. In our study, **the bacteria are purified from a naturally lysed amoebal culture. The fraction of reticulate bodies at this stage of infection is very low. Moreover, the gastrographin gradient allows a separation of EBs from RBs therefore further reducing the amount of RBs in the final product. We do not disrupt any non-lysed cells. A description of the purification protocol was added to the material and methods section.**

There are hardly any bacteria inside the cells in the image for C. sequanensis (Figure 3C); an image showing intracellular bacteria should be provided.

The whole paragraph on bacterial shape within cells was removed as proposed above.

Are the fixation procedures evaluated here the same as those in previous studies describing star and crescent shaped cells? This should be discussed.

The fixatives used were based on the study of Lindsay et al., 1995 and comparing the fixatives and buffer to the protocol used currently in our lab with PBS. To explain more why we used these buffers the following sentence was added to the introduction: "In this study the authors describe the presence of crescent shaped planctomycetes upon fixation with certain buffer and fixative combinations. "

The abstract should end with "... intracellular structures". The remaining two sentences do not discuss data from this manuscript and distract from the main findings.

As requested by the reviewer the last two sentences were removed.

The results section is largely redundant with the introduction/methods (the first two paragraphs, the first half of "Morphology of Chlamydiales within cells") and should be condensed.
The results section is mostly redundant with data presented in Table 2, and very difficult to read. Page 9 and 10 can be omitted almost entirely, i.e. should be reduced to avoid redundancy with Table 2.

The result section was reviewed to reduce redundancy with the introduction and the Table 3.

Supplementary figure 3 and details on OmcA are not related to the present study; can be omitted.

As requested by the reviewer the supplementary figure on OmcA was removed and the discussion was changed accordingly. "All bacteria analyzed in this paper encode for the large cysteine-rich outer membrane protein (OmcB), as well as an OmcA homolog. As mentioned by Collingro et al., (2011), *omcA* genes cannot be detected by simple homology searches. However, we were able to detect an *omcA* homolog by screening the upstream sequence of *omcB* for ORFs."

Reviewer #2: Data are presented on the ultrastructure of cells of members of the phylum Chlamydiae concerning the effect of chemical fixation conditions on the occurrence of unusual shapes which have in the past been observed especially in the Chlamydia-like bacteria. The authors deduce from their quite appropriate statistical analysis of their results that fixative and buffer conditions influence the proportion of cells with unusual shapes such as crescent and star, and these conclusions are justified. However the authors make another conclusion that is much weaker, based on the assumption that crescent and star shapes are artifacts, that a fixation method must be judged by its ability to preserve a round shape. The use of control Chlamydia seems to indicate that such methods are capable of preserving a round shape in some species not known to possess elementary bodies or other forms with crescent or star shapes, so that the question about the fixative conditions thatcould equally be posed is how effective they are in preserving crescent or star shapes, and the authors should consider this alternative in their Discussion.

Indeed we are not sure if these are really artifacts. We thus modified the discussion as follows: "While we consider the possibility that these shapes are artifacts the different patterns of morphologies

certainly seem to be consistent with differences in the composition and the organization of the proteinaceous layer and membrane between different *Chlamydia*-related bacteria."

Bioinformatic analysis suggesting explanations for differences in membrane proteins which might explain differences in shape after fixation are useful though there is no direct link made with localization of such proteins to a definite region e.g. via immunogold. However, the major question which anyone reading this paper familiar with recent papers is likely to ask is - where is a comparison with cells which have been cryofixed? That is , via for example cryosubstitution, cryosectioning, freeze-fracture? This might be expected for any other bacteria, but there may be good technical reasons why this has not been done for chlamydiae connected with their pathogenic nature and biohazards involved with their preparation for cryofixation. This should be clearly stated in the Introduction or Discussion since it is not clear to readers otherwise as to why chemical fixation is relevant at all. It is cryomethods which are now generally accepted to give rise to fewer artefacts regarding ultrastructure. There is a way this paper can still be acceptable in more or less present form - a sentence should be added to the final paragraph of the Discussion along these lines -
'However for cryotechniques to be applied, some technical challenges remain to be solved for freezing of live chlamydial pathogens without biohazard. Mild chemical fixation (e.g. with 0.3% glutaraldehyde) may be needed to ensure non-viability of cells before processing via cryofixation, and the results presented here may help the choice of such fixation conditions. Application of cryotechniques performed with the assistance of such knowledge should then resolve the question of the genuine existence of differences in morphotypes within species of Chlamydiales.'

First tests with cryofixation and deep-freezing are currently ongoing, but still in the development phase and will therefore be published in a further publication. As requested by the reviewer the following sentence was added to the discussion: "However, biosafety issues will still require a mild fixation step prior to cryofixation to prevent dissemination of infectious elementary bodies."

The authors should use line numbering in the next version to make it easier for review comments on the relevant section.

Line numbering was added to the manuscript.

Minor corrections:- all these corrections should be carried out in the revised version

Abstract:
Line 1, change 'with' to 'alternating between'
line 2 change 'form' to 'forms'
line 6: change 'buffers' to 'buffer' and 'fixatives' to 'fixative'
line 7: change 'combination' to combinations'
line 14: change 'considered' to 'applied' (this needs to be more definite than 'considered'
insert 'members' after 'Chlamydiales'

The suggested corrections were integrated in the abstract.

line 9: insert 'and designed' after 'images'

The parameters were not designed but combined. For clarity we added "combined".

line 17: when 'membrane' is referred to, do you really mean 'cell wall'?? This is at present confusable with cytoplasmic membrane, which I am not at all sure is meant here.

***Chlamydiales* do not possess a cell wall but a proteinaceous layer. The synthesis of the peptidoglycan is incomplete**. **This information can be found in the discussion, but for clarity it was mentioned in the introduction as well: "The *Chlamydiales* do not encode for a complete set of genes for the cell wall synthesis.** It is therefore believed that the structure of the bacteria is given by the network of cysteine-rich membrane proteins"

p.3 line 1: insert 'within phylum Chlamydiae of the PVC superphylum' after 'order' (this is after all a special issue about the superphylum)

The sentence was changed accordingly.

line 4: after 'related to' insert 'but relatively distant from'

change 'in Chlamydia' to 'in genera Chlamydia and Chlamydophila and other organisms in family Chlamydiaceae of order Chlamydiales'

> ***Chlamydia*-related bacteria is a descriptive term for all members of the other families that are part of the *Chalmydiales* order and that do not belong to the *Chlamydiaceae*. It would therefore not be appropriate to add 'but relatively distant from' in this context. According to the most recent decision of the taxonomy committee of the *Chlamydiales,* the *Chlamydiaceae* are not separated between *Chlamydia* and *Chlamydophila*.(Bavoil et al. 2013; Greub 2010)**

para 2: line 1: does Chlamydia refer to only genus Chlamydia here"

> **This statement refers to the whole *Chlamydiaceae* family and was changed accordingly.**

insert 'cells' after 'Chlamydia'

> **In the chlamydial field the terms elementary body and reticulate body are preferred to cells to avoid confusion with the host cell.**

para 2, line 10, insert 'as elementary bodies' after 'bodies'
line 11: are these sizes diameters? I any case replace 'large' by 'in size' in bth cases where this is used
delete 'as elementary bodies'

> **Since crescent bodies are not round we cannot speak of diameter, but we changed the sentence for more clarity "**Crescent bodies were estimated to be of the same size as elementary bodies (0.5μm in size), while reticulate bodies had a diameter of about 0.6μm."

p 4: para 1, line 7: change 'supported' to 'suggested'

> **The sentence was modified as follows: "**This hypothesis was previously described in a report by Lindsay et al. on the impact of fixative and buffer on Planctomycetes morphology (Lindsay 1995)."

para 2 line 4: delete 'and that'
start new sentence 'They probably do not correspond to additional developmental stages but rather are more likely to reflect differences ╔'

> **The sentence was modified as follows**: "These shapes probably do not correspond to additional developmental stages but are more likely to reflect differences in the underlying bacterial membrane protein composition or organization".

page. 5:
what resin was used? E.g. Spurr's, Epon, LR White? More detail is needed here

> **As requested by the reviewer the details on the resin were added in the material and method section.**

how were the cells grown? How can frozen bacteria be not subject to possible distortion in shape due to freezing?

> **For clarity the following sentence was added: "**Bacteria were produced by infection of *A. castellanii*".

Can your methods be justified on past experience with chlamydiae or the necessities of methods to grow them? This needs some explanation - no one would grow E.coli and then not fix them until after freezing. Maybe I have misunderstood some step or some step has been left out here.

> **The production of *Chlamydiales* requires the infection of a large amount of cells for a quite low yield. Moreover, to reach a high concentration of infected cells the bacteria must be expanded for up to three weeks. Usually we produce a stock of bacteria in a large amount and then store them frozen in 10% glycerol or SPG for further use. For these reasons we used frozen bacteria and not bacteria from freshly infected amoebae. Also the purification of these bacteria requires ultracentrifugation over a gradient. We cannot exclude that the bacterial shape was not affected by the freezing, but the bacteria are washed and "regenerated" in buffer prior to fixation. This information has been added to the material and method section.**

line 5: Insert 'Images' of ' before 'sections'

> **With the term "sections" we are referring to the slices of the ultrotome.**

line 6: supply kV used for operating TEM

> **The information was added in the methods section.**

para 2:line 1 - 'is 'as described above' meant here rather than 'as preiously described'??

The sentence was modified accordingly: “Samples were then dehydrated using acetone and embedded in an epoxy resin as described previously (Casson, 2006 #87).”

the species used need to be named here

The species used are named in the first paragraph of the methods.

line 2: change 'stocked' to'stored'
line 3: in insert in PBS' after 'glycerol'
insert 'and' after 'thawed'
line 13: 'dehydrated'
line 14: change 'formwar' to 'formvar'
line 15: insert 'and examined via TEM' after 'stained'

Changes were integrated as requested.

line 3- what is meant by 'one or two'?

Since not all the bacteria grow with the same efficiency in amoeba we sometimes used 2 vials of frozen bacteria to be sure to have enough material for electron microscopy image acquisition.

Several paragraphs were removed upon request of the first reviewer to reduce the complexity of the result section. Some of the changes were therefore not integrated in the manuscript since the sentences were completely removed.

page 6:para 1:
line 1: insert 'of sectioned cells or elementary bodies' ⇊after 'images'
line 2: change 'Further the images were' to 'The images were further'

Changes were integrated as requested.

para 2:line 2: add the following or something comparable after ⇊'results': 'in order to assess significant difference in proportions of morphotypes'

The sentence was changed as follows: “Unpaired t-tests with Welch’s correction were conducted on all results to determine significant differences of shape distribution.”

para 3: line 2: change 'were' to 'have been'

Since these results are already published they are considered to be true and should therefore not be referred in the past tense.

page 7: para 2: line 2: delete 'been'
line 5: change 'eyes' to 'eye'
line 9: give a reference and website to ImageJ
para 3: line 6: change 'amount' to 'number'

The requested changes were integrated in the text.

page 8:line 1: change 'amount' to 'number'
line 2:insert comma after 'one'
change 'less' to fewer'

The requested changes were integrated in the text.

what is meant by 'particles'? Define if possible - are these any cellular entities or only those compatible in size and other features with chlamydiae?

For clarity “particles” was replaced with “EBs”.

Line 3: insert 'as the pre-fixative' (or 'initial fixative'?) if appropriate

It’s not clear to us what the reviewer means by initial fixative. We preferred not the change the present formulation.

Para 2: line 5: with respect to what total is the percentage in relation to?

For clarity the following sentence was added: “The percentage was calculated as for the fixatives by normalizing by the number of particles acquired and the dilution of the sample.”

Para 3:line 1: change 'amount' to 'proportion'

The requested changes were integrated in the text.

Line 2: insert 'proportion of' before 'different shapes'
The requested changes were integrated in the text.

Line 4: change 'less' to 'fewer'
The requested changes were integrated in the text.

Page 9:line 2: change 'shape' to 'shapes'
Line 3: is 'proportion' rather than 'number' meant here?
The requested changes were integrated in the text.

Line 6: change 'less' to 'fewer'
The requested changes were integrated in the text.

Para 2: last line (8): change 'less' to 'fewer'
The requested changes were integrated in the text.

Para 3, line 1: change 'for' to 'with respect to the proportion of'
The requested changes were integrated in the text.

Line 6: insert 'the proportion of' after 'in'
The requested changes were integrated in the text.

Para 3: a second aim is mentioned, but it is not clear where the first aim is mentioned in this series - this should be more clearly indicated
Indeed we did not state the first aim in a very clear way we therefore changed the sentence in the beginning of the result sections as follows: "In a first step, we determined the effect of the fixative on the different shapes the same buffer conditions were compared when applicable (Table 2)."

Page 10" para 2, line 1: change 'bacteria' to 'species'
The requested changes were integrated in the text.

Delete 'an'

Line 8: change 'to the profit' to 'in favour'
The requested changes were integrated in the text.

Second last line: change 'amount' to 'proportion' if appropriate
Make more clear what 'undetermined' means - could not be classified into round, star or crescent?
The changes suggested by the reviewer were added in the manuscript. A better description of the undetermined was added in the beginning of the result section to read as follows: "The undetermined small shape generally corresponded to small elementary bodies with an irregular shape or bacterial debris. The large undetermined shapes were often constituted of two bacteria too close to each other to allow distinct outline recognition."

Page 11: line 8: change 'a quite' to 'an'
The requested changes were integrated in the text.

Line 9: change 'particles' to 'particle categories'
The requested changes were integrated in the text.

Para 2. Line 1: insert 'when fixed chemically' after 'Chlamydiaceae'
Line 4: change 'like' to 'such as'
Lne 12: close up 'over night'

Re para 2:
Doesn't the observation that no stars or crescents were observed with C pneumomiae are fixed with the same protocol as for the chlamydia-like organisms indicate that the crescent and star shapes are not in fact artefacts?
In other words. C pneumoniae acts as a control here
Page 12: line 1: it is an assumption surely that different shapes from round are induced by different fixatives - there is no real control here other than different species can supply. The possibility should be considered that different fixatives could be preserving different shapes with different degrees of effectiveness rather than 'inducing' them

Upon request by the first reviewer the chapter on bacteria within infected cells was removed.

Discussion - there should be some statement that the methods applied here will form the basis for assessing morphological differences when chlamydiae are eventually examined by cryosubstitution - e.g. in testing a hypothesis that chemical fixation induces such shapes

At the end of the discussion we added a sentence on the importance of cryofixation for confirmation of these morphologies: "The occurrence of these unusual shapes of EBs should be investigated using cryofixation or deep-freezing methods that ideally preserve bacterial integrity to a higher extent."

Page 12, Para 2 line 4 - change 'to a varying degree for peptidoglycan biosynthesis' to 'genes for peptidoglycan biosynthesis to a varying degree'

The sentence was changed accordingly

What is meant by 'these bacteria? Ambiguity here vsinceplanctomycetes and chlamydia mentioned next sentence

As suggested by the reviewer the sentence was clarified.

Line 9: change 'Chlamydia' to 'Chlamydiae'

As suggested by the reviewer the sentence was modified.

Para 3: line 2: add 'in the phylum Clamydiae' after 'bacteria'

As suggested by the reviewer the sentence was modified.

One assumes confocal laser scanning microscopy was used in fluorescence mode? If not, state this

As suggested by the reviewer the sentence was clarified.

Line 4: change 'display' to 'displays'

Since there are several bacteria we used the plural form.

Line 6: insert 'different' after 'Using', and change 'fixation' to 'fixations' if appropriate

As suggested by the reviewer the sentence was modified.

Page 13: line 3: why preferential? Assumes round shapes as non-artifactual - but what if osmium tetroxide induces round cell shape in crescent and star forming species??

Indeed we cannot know yet which form is the natural form, we therefore removed the statement from the discussion.

Para 2:give a reason why Hepes buffer is supposed to be 'good' - what does this mean" Good in what way? Better preservation? Fewer artefacts??
Try to use better word than 'good'

As suggested by the reviewer the sentence was clarified.

Para 3: line 5: change 'like' to 'as with'
Line 6: change 'amount' to 'proportion'

As suggested by the reviewer the sentence was modified.

Line 8: change 'appears' to 'may' -
In addition - the proposition in this sentence is by no means proven without a cryofixation method control
Line 9-10: rather than causing membrane leakage, glutaraldehyde is more likely to cross-link cytoplasmic membrane to wall making shrinkage to a crescent more likely under hyperosmotic buffer conditions. The mechanism of glutaraldehyde fixation is well-established as protein cross-linking. See Lindsay et al 1995 reference in your bibliography

The paragraph was rewritten to take into account the comments of the reviewer.

Line 11: change 'amount' to 'proportion'

As suggested by the reviewer the sentence was modified.

Para 4: insert 'the proportion observed is ' after 'Although'

As suggested by the reviewer the sentence was modified.

Insert 'this effect' before 'observed'

As suggested by the reviewer the sentence was modified.

Page 14:

Line 2: change 'confirms' to 'is consistent with' (it does not confirm the observations since these are using completely different methods and approaches)

As suggested by the reviewer the sentence was modified.

Para 2: line 10: reduced relative to what species?

As suggested by the reviewer the sentence was clarified.

Line 15: delete 'as'

As suggested by the reviewer the sentence was modified.

Line 16: change 'results' to 'result'

As suggested by the reviewer the sentence was modified.

Line 18: the precedence for unusual cell shapes in bacteria should be noted e.g. even with phase contrast microscopy of live cells bacteria such as Ancalomicrobium, Prosthecomicrobium, Prosthecobacter, and Caulobacter exhibit extensions (called prosthecae) to the cell cytoplasm which involve wall as well as cytoplasm of the cells - they are not fixation artefacts since they are observed in live cells as well as fixed cells. See for example Staley JT. Prosthecomicrobium and Ancalomicrobium: new prosthecate freshwater bacteria. J Bacteriol. 1968 May;95(5):1921-42. Also in verrucomicrobia within the PVC superphylumcryofixed cells still show extensions and unusual cell shape even after cryofixation rather than chemical fixation, following original phase contrast observations when they were originally described e.g. Verrucomicrobiumspinosum and Prosthecobacterdejongeii. See Lee KC, Webb RI, Janssen PH, Sangwan P, Romeo T, Staley JT, Fuerst JA. Phylum Verrucomicrobia
representatives share a compartmentalized cell plan with members of bacterial phylum Planctomycetes. BMC Microbiol. 2009 Jan 8;9:5.

The information provided by the reviewer was added in the discussion as follows: "Among others, cryofixed *Verrucomicrobia* present an elongated shape and spikes (Lee et al. 2009). Star-shaped bacteria were also observed by phase-contrast and electron microscopy of freshwater samples (Staley 1968). However, the exact nature of these bacteria is not known since they were observed in a mixed culture from a river isolate."

Para 3 line 2: insert 'though not in Chlamydia pneumoniae' after 'species'

This sentence was removed.

Insert 'if only chemical fixation methods are used' before 'detection'

The sentence was modified as follows: "Therefore, if only chemical fixations are used, presence of a given shape in any new isolate should be interpreted with caution to classify at the family level, and other criteria such as gene sequences should be used for taxonomy (Greub 2010)."

Page 15:Line 1: insert 'seem to be consistent with or' before 'reflect'

The sentence was changed accordingly: "While we consider the possibility that these shapes are artifacts the different patterns of morphologies certainly seem to be consistent with differences in the composition and the organization of the proteinaceous layer and membrane between different *Chlamydia*-related bacteria."

Lines 3-4: 'variations .' ; this is really too speculative and should be deleted - it does not reflect the data presented with do not clearly point to this in a definite way

Line 7: delete 'now'

The suggested changes were integrated in the manuscript.

Line 8: add a sentence along the lines of ' However, some technical challenges may remain to be solved for freezing of live pathogens without biohazard. Mild chemical fixation ma be needed (e.g. using very dilute glutaraldehyde) to ensure non-viability before processing, and the results presented here may help choose such fixation conditions' - without such a sentence the reader may well ask - why was such a classical and rather out-dated technique for TEM preparation as chemical fixation used at all by the authors? Some explanation must certainly be given as to why chemical fixation was investigated in absence of cryosubstitution or another cryotechnique e.g. cryosectioning, freeze-fixation etc.

As suggested by the reviewer we added the information accordingly (see above).

Page 21:Fig 1 legend: What does 'undetermined' mean? Does it mean 'Could not be determined clearly as crescent or star or round?' Give some key to this in legend text. What is criterion for distinction between 'small' and 'large' can you give one in micrometres?

Since the shapes are irregular there cannot be a cutoff in micrometers. The unclassified large shapes were selected according to visual inspection of two bacteria too close to each other for single outline recognition. For the small unclassified, the shape could not be determined and was therefore considered to be unclassifiable. The classification for undetermined was explained in the main text and was also added to the figure legend.

Fig 2 legend: what is the percentage relative to -⏚- i.e. what would be 100%?? % does not make sense here

It represents the proportion of particles in a particular buffer and fixative condition compared to the total amount of elementary bodies analyzed for each bacteria. For more clarity the legend was changed as follows: "Quantification of bacterial morphological features depending on fixation**.** (**a**) Percentage of bacteria in sample per fixation condition. The amount of elementary bodies (EBs) detected in each fixation and buffer condition were compared to the total amount of EBs quantified for each bacterium. Loss of bacteria was observed with 3% glutaraldehyde in almost all bacteria. (**b**) For each bacterium the percentage of each shape is represented according to fixation method (compared to the total amount of EBs for each condition and bacteria)."

ESM Fig 2 legend: line 2: insert 'fixation of bacteria in' before '100mM'
Delete 'fixed bacteria'
Line 3: change 'statistical' to 'statistically'

The legend was adapted accordingly.

What does 'undetermined' mean? Could not be determined clearly as crescent or star or round? Give some key to this in legend text

The classification for undetermined was explained in the main text as follows: "The undetermined small shapes corresponded to small elementary bodies with an irregular shape. The large undetermined shapes were two bacteria too close to each other to allow distinct outline recognition."

Page 22:line 1: insert 'of morphotypes' after 'analyses'

Changes were introduced as requested

ESM Fig 3 legend, line 4: insert 'of sequence positions is' after 'Numeration'

This figure was removed form the manuscript as requested by the first reviewer.

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Crescent and Star Shapes of Members of the *Chlamydiales* Order: Impact of Fixative Methods

Brigida Rusconi[1*], Julia Lienard[1*], Sébastien Aeby[1], Antony Croxatto[1], Claire Bertelli[1,2], Gilbert Greub[1]

1 Center for Research on Intracellular Bacteria, Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

2 SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland

* These authors contributed equally to this work

Corresponding author

Prof. Gilbert Greub
Institute of Microbiology
Rue du Bugnon 48
1011 Lausanne
Switzerland
Tel: 0041 21 314 4979
Fax: 0041 21 314 4060
E-mail: gilbert.greub@chuv.ch

Running title

EM Morphology of *Chlamydia*-related bacteria

Keywords

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ABSTRACT

Members of the *Chlamydiales* order all share a biphasic lifecycle alternating between small infectious particles, the elementary bodies (EBs) and larger intracellular forms able to replicate, the reticulate bodies (RBs). Whereas the classical *Chlamydia* usually harbours round-shaped EBs, some members of the *Chlamydia*-related families display crescent and star-shaped morphologies by electron microscopy. To determine the impact of fixative methods on the shape of the bacterial cells, different buffer and fixative combinations were tested on purified EBs of *Criblamydia sequanensis, Estrella lausannensis, Parachlamydia acanthamoebae*, and *Waddlia chondrophila*. A linear discriminant analysis was performed on particle metrics extracted from electron microscopy images to recognize crescent, round, star and intermediary forms. Depending on the buffer and fixatives used, a mixture of alternative shapes were observed in varying proportions with stars and crescents being more frequent in *C. sequanensis* and *P. acanthamoebae,* respectively. No tested buffer and chemical fixative preserved ideally the round shape of a majority of bacteria and other methods such as deep-freezing and cryofixation should be applied. Although crescent and star shapes could represent a fixation artifact, they certainly point towards a diverse composition and organization of membrane proteins or intracellular structures rather than being a distinct developmental stage.

Introduction

Chlamydiales, which belong to the *Chlamydiae* phylum of the *Planctomycetes, Verrucomicrobia, Chlamydia* (PVC) superphylum are obligate intracellular bacteria that have been isolated from a variety of clinical and environmental samples (Lienard and Greub 2011; Horn 2008). The term *Chlamydia*-related bacteria has been used to coin a variety of bacterial species that belong to several families phylogenetically related to the well-known pathogens *Chlamydia.* These bacteria have the ability to infect a wide range of hosts and cell lines,

some being able to grow within amoebae (Greub and Raoult 2004; Corsaro et al. 2009), human cell lines (Goy et al. 2008; Kebbi-Beghdadi et al. 2011b), arthropods (Corsaro et al. 2007) or fishes (Kebbi-Beghdadi et al. 2011a). However, members of the *Chlamydiales* all share a unique life cycle. Their infectious EB is internalized into the host cell within a membrane-bound vacuole termed an inclusion. Soon after entry, the EB differentiates into a RB and divides by binary fission. Finally, RBs re-differentiate into EBs and lyse the host cell to start a new infection cycle.

Chlamydiaceae usually harbour round-shaped EBs that are small in size (0.3-0.35 µm) as well as slightly larger RBs (0.5-2.0 µm) (Mitchell et al. 2009; Miyashita et al. 2001). These round shapes were confirmed by freeze deep-etching (Matsumoto et al. 1976). The *Chlamydiales* do not encode for a complete set of genes for the cell wall synthesis (McCoy and Maurelli 2006). It is therefore believed that the structure of the bacteria is given by the network of cysteine-rich membrane proteins. During the past decade, particular EB cell morphologies were described for new members of the *Chlamydiales* order. An electron micrograph study of *Parachlamydia acanthamoebae* in the amoeba *Acanthamoeba polyphaga* showed the occurrence of crescent shapes mainly within amoebal inclusions (Greub and Raoult 2002). This latter form, also seen with other members of the *Parachlamydiaceae* family (Amann et al. 1997; Horn et al. 2000), was proposed as a potential third replicative stage. This stage exhibits similar biological characteristics to elementary bodies, being an infectious stage that enters in amoebae passively by phagocytosis and that accumulates in vacuoles full of elementary bodies at late time points (Greub and Raoult 2002). Crescent bodies are estimated to be of the same size as elementary bodies (0.5 µm large), while reticulate bodies are about 0.6 µm large.

Subsequently, a new chlamydial species called *Criblamydia sequanensis* was discovered using amoebal co-culture of a water sample from the Seine river (Thomas et al. 2006). This bacterium exhibited an unusual star-shaped EB and an oblong lamellar structure within its cytoplasm. Recently, *Estrella lausannensis* was described as a new species harbouring

similar phenotypic characteristics, except for the absence of the translucent lamellar structure (Lienard et al. 2011). The star-shaped morphology of EBs of *C. sequanensis* and *E. lausannensis* was proposed as a distinctive characteristic of the *Criblamydiaceae* family (Lienard et al. 2011). As for *Parachlamydia*, EBs were shown to be slightly smaller than RBs, with sizes ranging between 0.5-1 µm and 0.8-1.8 µm, respectively. The crescent and star shapes might also appear following use of fixative and buffer during the preparation of the embedded electron microscopy samples. This hypothesis was previously described in a report by Lindsay et al. on the impact of fixative and buffer on *Planctomycetes* morphology (Lindsay 1995). In this study the authors describe the presence of crescent-shaped *Planctomycetes* upon fixation with certain buffer and fixative combinations.

In this contribution, we show the effect of different fixation methods for electron microscopy on the morphology of four bacteria across three different families of the *Chlamydiales* order: *Waddliaceae*, *Parachlamydiaceae*, and *Criblamydiaceae*. We establish that the proportion of crescent and star shapes are dependent on fixatives and buffers used for sample preparation. These shapes probably do not correspond to additional developmental stages but are more likely to reflect differences in the underlying bacterial membrane protein composition or organization.

Material and methods

Strains

The following bacterial strains were used: *Waddlia chondrophila* (ATCC VR-1470), *Criblamydia sequanensis* (CRIB18), *Parachlamydia acanthamoebae* (Hall's *coccus*), and *Estrella lausannensis* (CRIB30). These bacteria were propagated in *Acanthamoeba castellanii* (ATCC30010).

Preparation of purified elementary bodies

The following fixation conditions were tested on elementary bodies purified as previously described (Greub et al. 2003b). Briefly, bacteria were produced by infection of *A. castellanii.* Lysed culture was centrifuged at 180g to remove amoebal debris. Then bacteria were pelleted at 6'600g. Bacteria were washed in a PBS, 10% sucrose solution and centrifuged as previously reported (Greub et al. 2003a). The elementary bodies were then separated by ultracentrifugation with a discontinuous gastrographin gradient. Purified bacteria were stored frozen at -80°C in 10% Glycerol in PBS or succinic, phosphate, glycine (SPG) buffer prior to fixation. One or two vials of frozen bacteria (1ml) were thawed and centrifuged for 10min at 7'500g. Bacteria were washed with 3mM Hepes and split in two samples prior to centrifugation. Pellets were resuspended in 300µl of 100mM PBS or 3mM Hepes. The samples were centrifuged again and resuspended in the same buffers. The samples resuspended in 3mM Hepes were used for the two Hepes conditions (3mM and 100mM). After overnight fixation of all the samples with the corresponding fixative (1% osmium tetroxide or 3% glutaraldehyde) at 4°C, the samples were washed three times with the corresponding buffer: 3mM or 100mM Hepes or 100mM PBS. After a PBS wash, cells were further fixed with 1% osmium tetroxide in PBS for 1h at room temperature. Samples were dehydrated with subsequent increasing ethanol washes (50-100%). Samples were then transferred into propylene oxide and incubated over night in an epoxy resin (Epon) mixed with 50% propylene oxide as described previously (Casson et al. 2006). Samples were embedded in agar capsules. Thin sections obtained with the LKB 2088 Ultrotome were deposited on formvar coated copper grids and stained. Sections were stained with methanol-uranyl acetate and lead nitrate with sodium citrate for 10min. Sections were acquired with a transmission electron microscope with a 80kV filament (Philips EM 201). Thus, a total of 6 conditions were investigated, i.e. 3 buffers (100mM Hepes, 3mM Hepes, 100mM PBS) and two different fixatives (1% osmium tetroxide, 3% glutaraldehyde). Osmolarity of the different buffers can be found in supplementary Table S1. The osmolarity was measured with a 2020 Osmometer from Applied Instruments (Vlissingen, Netherlands).

Image analysis

Electron microscopy images of elementary bodies taken at 7000x magnification were transformed in a mask with the function “Make Binary” of ImageJ (Schneider et al. 2012). The images were further analyzed with the “Analyze Particle” plugin to extract a set of parametric values (Supplementary Table 2, Supplementary Fig. 1) that characterize each particle. To exclude potentially remaining reticulate bodies and aberrant forms, particles outside the following circularity and size cutoff were excluded (Circularity: 0.2-1, size: 0.02-0.15 pixel^2). To control for bacterial aggregation problems the mean area was determined by quantifying the mean particle size in images without size filter.

To identify the combination of ideally discriminating parameters for each type of shapes (crescent, star, convex polygon, round, undetermined large, undetermined small), a set of 488 particles was visually and manually selected. Each particle was assigned randomly to a training set (200 particles) or to a test set (288 particles), implying that training and test sets are non-redundant. A linear discriminant analysis (LDA) was trained on the training set. Then, the LDA was used to predict the shape of the test set allowing to measure the accuracy of shape assignment. Finally, each picture was automatically processed through ImageJ and LDA analysis respectively to evaluate the proportion of each shape. During the LDA analysis, all objects recognized as a valid particle by ImageJ were classified in either one of the shapes.

In addition, to assess the global performance of LDA on varying training sets, 100 random training sets and their complementary test sets were used to train 100 LDA and determine in each case the specificity and sensitivity of shape assignment. LDA analyses were performed using R (RCoreTeam 2012) and package MASS (Venables and Ripley 2002).

Statistical analysis

Statistical analysis of morphology quantification was performed with GraphPad Prism v6.0 (GraphPad, LaJolla, USA). Unpaired t-tests with Welch’s correction were conducted on all

results to determine significant differences of shape distribution. Correlations were calculated using the Pearson correlation coefficient.

Results

Computer-based determination of bacterial morphology

In the *Chlamydiales* order several different morphologies were previously described (Greub and Raoult 2002; Lienard et al. 2011; Thomas et al. 2006; Corsaro et al. 2007). We investigated the role of two fixatives and three buffers chosen accordingly to Lindsay et al., 1995 on the morphology of 4 *Chlamydia*-related species *C. sequanensis*, *E. lausannensis* (*Criblamydiaceae*), *W. chondrophila* (*Waddliaceae*), and *P. acanthamoebae* (*Parachlamydiaceae*) (Table 1). In our study we defined the following shapes: crescent, star, convex polygon, round, undetermined large and undetermined small (Fig. 1a). The undetermined small shape generally corresponded to small elementary bodies with an irregular shape or bacterial debris. The large undetermined shapes were often constituted of two bacteria too close to each other to allow distinct outline recognition.

Determination of bacterial morphological features by computer-assisted analysis of images has proven to be quite challenging. The human eye has a very unique ability to readily and reliably detect different complex shapes within a picture. A collection of shapes selected by eye were used to define finite parameters for computer-based analysis that allow the automated classification of each particle within any picture to each shape.

Thirteen parameters that define different metrics of particle shape and size for each pre-determined population were acquired. Simple combinations of two parameters were not sufficient to discriminate between all shapes (Fig. 1b, supplementary Fig. 1). Therefore, we performed a linear discriminant analysis (LDA) that attempts to express the shape category as a linear combination of all available parameters (Fig. 1c). The LDA was trained on a set of

200 manually selected particles and then tested against a different set of 288 particles to assess the accuracy of shape classification (Table 2). An analysis of 100 random training sets and the 100 complementary test sets among the 488 particles manually selected achieved a mean specificity above 96% and a mean sensitivity ranging between 75% for crescent shape and 97% for round shape (Fig. 1d).

Effect of fixatives and buffers on the number of particle analyzed

Including all fixative and buffer conditions tested, we analyzed a total of 91'062 particles. The sample density was low enough to allow recognition of different shapes in each buffer and fixative conditions. For *C. sequanensis* and *E. lausannensis,* fixation with 3% glutaraldehyde and 100mM Hepes caused a severe lysis of the bacteria that did not allow the acquisition of enough particles for analysis (Table 1). The same lysis occurred with *P. acanthamoebae* in 3% glutaraldehyde 100mM PBS (supplementary Fig. S3). To rule out that aggregation of bacteria significantly leads to a reduced number of particles counted, we quantified the mean particle area for each condition (supplementary Fig. S2). For none of the bacteria, we could observe a correlation between the mean area and the initial bacterial concentration. However, for *W. chondrophila* and *C. sequanensis* the area was significantly higher for 100mM PBS in 1% osmium tetroxide compared to all other investigated conditions (Fig. S2).

To investigate the role of the fixative on the preservation of bacterial cells, the number of bacteria present in each sample was determined by normalizing the number of particles acquired with the number of images taken and the dilution of bacteria used for fixation (Fig. 2a). For all buffer conditions except one, significantly ($p<0.0001$) more EBs were observed with 3% glutaraldehyde compared to 1% osmium tetroxide. Only for *P. acanthamoebae* 100mM Hepes there were more particles with 1% osmimum tetroxide ($p<0.0001$). Subsequently, despite the higher proportion of particles acquired in 3% glutaraldehyde, one condition was causing the complete lysis of EBs in three out of four bacteria. Therefore, we determined the influence of the buffer on the lysis of the bacteria in 1% osmium tetroxide

fixed cells (Fig. 2a). Again, the percentage was calculated as the number of particles normalized by the number of images acquired and the dilution of the sample. For all bacteria there were significantly more particles analyzed with 100mM PBS compared to 100mM Hepes ($p<0.0001$) and with 3mM Hepes compared to 100mM Hepes ($p<0.0001$). When comparing 100mM PBS to 3mM Hepes the percentage of particles was still significantly higher with 100mM PBS for *C. sequanensis* ($p<0.0001$), *W. chondrophila* ($p<0.0001$), and *P. acanthamoebae* ($p<0.02$). Overall, the 100mM PBS buffer appeared to better preserve the bacteria.

Effect of fixatives and buffers on bacterial morphology

The LDA allowed us to quantify for each fixation the amount of each bacterial shape (Fig. 2b). In a first step, we determined the effect of the fixative on the proportion of different shapes by comparing the same buffer, when applicable (Table 3). For *P. acanthamoebae,* fixation with 1% osmium tetroxide with 3mM Hepes or 100mM Hepes reduced the number of crescent bodies compared to 3% glutaraldehyde. On the other hand, for the same buffer *W. chondrophila* showed an increase ($p<0.0001$) in crescent shapes with the 1% osmium tetroxide fixation. For the phosphate buffer (100mM PBS) only *C. sequanensis* exhibited a significant decrease ($p=0.0005$) of crescent shapes with the 1% osmium tetroxide fixation. In summary depending on the bacterial species, an increase or decrease in the number of crescent shapes was observed with the different fixatives.

We saw an increase in star shapes with 3mM Hepes / 1% osmium tetroxide compared to 3% glutaraldehyde for *E. lausannensis* ($p<0.0001$) and *W. chondrophila* ($p<0.0001$). In contrast, *C. sequanensis* ($p<0.0001$) and *P. acanthamoebae* ($p=0.0097$) presented a decreased proportion of star shapes in the same conditions (Table 3). Overall, we observed fewer star shapes with 1% osmium tetroxide.

When comparing fixatives for round-shaped bacteria a decrease with both 3mM Hepes / 1% osmium tetroxide ($p<0.0001$) and 100mM PBS / osmium tetroxide ($p<0.0001$) was observed

for *C. sequanensis* compared to 3% glutaraldehyde. For *E. lausannensis* (p=0.0003) and *W. chondrophila* (p<0.0001), we also observed a reduced proportion of round-shaped bacteria with 3mM Hepes / 1% osmium tetroxide and 100mM Hepes / 1% osmium tetroxide, respectively. Only for *P. acanthamoebae* an increase in round-shaped bacteria with both 3 and 100mM Hepes / 1% osmium tetroxide (p<0.0001) was observed (Table 3). In summary, we again observed different changes in shapes depending on the bacterial species.

The second aim of our study was to determine the role of the different buffers on the morphology of the bacteria. The percentage of crescent-, star-, and round-shaped bacteria fixed with 1% osmium tetroxide according to the buffer used was determined (Fig. 2b). For *C. sequanensis* and *W. chondrophila* no significant change in the proportion of crescent shapes was observed with the three different buffers. For *P. acanthamoebae* there were more crescent-shaped bacteria in 100mM PBS compared to both 100mM Hepes (p<0.0001) and 3mM Hepes (p<0.0001). For *P. acanthamoebae* the change in buffer rather than the change in concentration affected the proportion of crescent-shaped bacteria (Table 3).

The proportion of star shapes were strongly associated with the use of an Hepes buffer, star shapes being present in lower numbers in 100mM PBS than in any concentration of Hepes buffer (Table 3). For all except *P. acanthamoebae* more round-shaped bacteria were observed in 100mM PBS compared to both 3mM and 100mM Hepes (Table 3). In summary, 100mM PBS reduced the proportion of star shapes in favour of more round-shaped bacteria. In general, the proportion of crescent-, star- and round-shaped *C. sequanensis*, *W. chondrophila*, and *E. lausannensis* changed in the same way when comparing different buffers. *P. acanthamoebae* on the other hand often showed an opposite behavior, i.e. a decrease of round-shaped bacteria with 100mM PBS compared to 100mM Hepes. Still, 100mM PBS appeared to be the best buffer, since the proportion of undetermined shapes was low for all bacteria in this condition (Fig. 2b). Conversely, the 100mM Hepes, 1% osmium tetroxide combination increased strongly the proportion of undetermined shapes for all four species.

Considering 100mM PBS with 1% osmium tetroxide to be the least aggressive and best preserving fixative we then compared the percentage of crescent, star and round shapes between different species. In this condition *P. acanthamoebae* presented the highest percentage (15.8%) of crescent shapes, followed by *E. lausannensis*, *W. chondrophila* and *C. sequanensis* that harboured less than 5% crescent shapes. More than a third (37.5%) of *C. sequanensis* particles were star-shaped, followed by *E. lausannensis* and *W. chondrophila* at about 20% and finally *P. acanthamoebae* that presented only 12% star shapes. The highest proportion (32%) of round-shaped bacteria was found in *E. lausannensis* followed by *W. chondrophila* (22%), *P. acanthamoebae* (9.5%) and finally *C. sequanensis* (2.4%). In summary, under these fixation and buffer conditions, *C. sequanensis* is mainly characterized by star shapes, *E. lausannensis* by round-shaped bacteria, *P. acanthamoebae* by crescent and convex polygons, while *W. chondrophila* had an equal proportion of star, round and convex polygon particles.

Discussion

Morphology of bacteria is strongly dependent on the composition of the cellular membrane and the cell wall. Bacteria with a peptidoglycan display a higher rigidity, preventing significant changes in cell shape and size. Members of the *Planctomycetes, Verrucomicrobia, Chlamydiales* (PVC) superphylum encode genes for peptidoglycan biosynthesis to a varying degree (Labutti et al. 2010; Stephens et al. 1998; Yoon et al. 2010). According to a post-genomic analysis of peptidoglycan biosynthesis based on three necessary genes (GT28, GT51, one of five GH family genes) both *Chlamydiales* and *Planctomycetes* do not synthesize peptidoglycan (Cayrou et al. 2012). Indeed, so far no peptidoglycan was ever isolated from *Planctomycetes* and *Chlamydiae* (Fox et al. 1990; Yoon et al. 2010). Still, for *Chlamydia pneumoniae* the peptidoglycan precursor lipid II was produced by chlamydial proteins (MraY, MurG) from the substrate UDP-MurNAc-pentapeptide *in vitro* (Henrichfreise et al. 2009). Moreover, *Chlamydiales* and some members of the *Planctomycetes* encode for

penicillin binding protein homologs that might replace the missing transpeptidase function of GT51. The role of this peptidoglycan precursor in chlamydial cell wall organization remains controversial.

In this study we investigated the effect of different fixatives and buffers on the cell shape of these peptidoglycan-less bacteria in the phylum *Chlamydiae*. Confocal microscopy of cells infected with *W. chondrophila, P. acanthamoebae*, or *E. lausannensis* and labeled with fluorescent antibodies, generally display round bacteria (Goy et al. 2008; Greub et al. 2005; Lienard et al. 2011), but crescent bodies have been observed following paraformaldehyde fixation of *P. acanthamoebae* (Greub et al. 2005). Bacteria were stored frozen at -80°C with 10% glycerol or in SPG prior to fixation. Although we cannot exclude that this step might influence the morphology of the bacteria, the bacteria are viable and infectious. Using different chemical fixations, we could observe a different proportion of shapes, including crescents and stars, for all bacteria analyzed. Interestingly, the peptide cross-linking agent glutaraldehyde caused a higher lysis of the bacteria than the oxidizing osmium tetroxide in combination with higher osmolarity buffers (100mM Hepes, 100mM PBS). Moreover, round-shaped bacteria were more frequently observed with osmium tetroxide.

Even though Hepes 100mM is considered to have an appropriate osmolarity range for natural shape conservation (Lindsay 1995) for electron microscopy we observed that PBS preserved better the round shape for our bacteria. On the other hand, *P. acanthamoebae* 3mM Hepes / 1% osmium tetroxide fixation resulted in more round-shaped bacteria than the other two buffers. For *C. sequanensis* none of the used fixatives or buffers resulted in more than 7% of round-shaped bacteria. Other buffer conditions, like cacodylate should be tested to determine a buffer with appropriate osmolarity. Rather than osmolarity the ion composition might influence *C. sequanensis* morphology by electrostatic interactions with the cell wall.

In *Gemmata obscuriglobus* (*Planctomycetes*) the combination of 3% glutaraldehyde and 3mM Hepes increased the amount of crescent-shaped bacteria detected compared to 1%

osmium tetroxide (Lindsay 1995). The same observation was made with *P. acanthamoebae* that showed the highest percentage of crescent shapes among the bacteria tested. Moreover, as with *G. obscuriglobus* an increase in osmolarity to 100mM Hepes with 3% glutaraldehyde increased the proportion of crescent shapes observed in *P. acanthamoebae*, although not to the same extent. This suggests that for *P. acanthamoebae* the fixative may play a major role in shape determination, probably influencing the membrane protein crosslinking. Glutaraldehyde could cause the crosslinking of the proteinaceous cell membrane to internal cell structures of *P. acanthamoebae* therefore increasing the alteration of bacterial morphology into crescent-shaped bacteria. This is also supported by the higher proportion of round bacteria with 1% osmium tetroxide compared to 3% glutaraldehyde with both Hepes buffer concentrations.

The formation of star shapes in *C. sequanensis* and crescent shapes in *P. acanthamoebae* is certainly triggered by some intrinsic differences in the membrane or proteinaceous layer of these bacteria. The different morphology of *Chlamydia*-related bacteria with the same fixative condition underlines the differences in membrane proteins determined by genome analysis (Bertelli et al. 2010; Horn et al. 2004) and proteomics (Heinz et al. 2010; Heinz et al. 2009; Lienard et al. 2013).

Presence and abundance of MOMP-like proteins varies significantly between *Chlamydia*-related bacteria, ranging from none in *Protochlamydia* to 35 in *Simkania negevensis* (Collingro et al. 2011). *W. chondrophila* and the two members of the *Criblamydiaceae* encode for about a dozen MOMP-like proteins. All bacteria analyzed in this paper encode for the large cysteine-rich outer membrane protein (OmcB), as well as an OmcA homolog. As mentioned by Collingro et al., (2011), *omcA* genes cannot be detected by simple homology searches. However, we were able to detect an *omcA* homolog by screening the upstream sequence of *omcB* for ORFs. Moreover, the polymorphic membrane protein (pmp) family is strongly reduced in *W. chondrophila, Protochlamydia amoebophila* and *S. negevensis* (Collingro et al. 2011; Bertelli et al. 2010) as well as in *E. lausannensis* (n=1) and in *C.*

sequanensis (n=2) compared to *Chlamydiaceae*. These differences in protein composition might partially explain the differences in cell shape observed for each species with the different fixatives. Uneven protein distribution combined with a reduced cross-linking of proteins by the fixative within the membrane may well result in “collapsing” parts. Finally, we cannot exclude the cross-linking of intracellular protein structures that are unevenly distributed inside the bacteria and could cause particular cellular shapes.

In summary, we observed that particular crescent and star shapes are observed in all *Chlamydia*-related species. Therefore, if only chemical fixations are used, presence of a given shape in any new isolate should be interpreted with caution to classify at the family level, and other criteria such as gene sequences should be used for taxonomy (Greub 2010).” However, differences in the proportion of chlamydial cell shape depend on the species, fixatives, and buffers. While we consider the possibility that these shapes are artifacts the different patterns of morphologies certainly seem to be consistent with differences in the composition and the organization of the proteinaceous layer and membrane between different *Chlamydia*-related bacteria. Unusual morphologies have been described in other bacteria. Among others, cryofixed *Verrucomicrobia* present an elongated shape and spikes (Lee et al. 2009). Star-shaped bacteria were also observed by phase-contrast and electron microscopy of freshwater samples (Staley 1968). However, the exact nature of these bacteria is not known since they were observed in a mixed culture from a river isolate. It is so far not possible to link this to the presence/absence of known outer membrane proteins. For each new *Chlamydia*-related bacterium the chemical fixation must be optimized to preserve as much as possible the natural shape of the bacteria. The occurrence of these unusual shapes of EBs should be investigated using cryofixation or deep-freezing methods that ideally preserve bacterial integrity. Only these techniques will allow to determine if these shapes are actually present in the natural state or induced by chemical fixation. However, biosafety issues will still require a mild fixation step prior to cryofixation to prevent dissemination of infectious elementary bodies.

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CONFLICT OF INTEREST

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors declare having no conflict of interest related to the content of this contribution.

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Table 1 Particles acquired per condition and bacterial species

Species	3% glutaraldehyde			1% osmium tetroxide		
	100mM Hepes	3mM Hepes	100mM PBS	100mM Hepes	3mM Hepes	100mM PBS
C. sequanensis	NA	7600	5306	6365	6960	5663
E. lausannensis	NA	1497	734	1087	2518	1160
W. chondrophila	8331	6805	6477	5095	4169	4568
P. acanthamoebae	2584	2675	NA	4615	4095	2758
Total	10'925	18'577	12'517	17'162	17'742	14'149

NA: not analyzed

Table 2 Confusion table of specificity and sensibility

Shape	True positive	False positive	True negative	False negative	Sensitivity	Specificity
Crescent	35	9	235	9	79.5	96.3
Round	51	1	234	2	96.2	99.6
Convex polygon	57	5	226	0	100	97.8
Star	54	8	223	3	94.7	96.5
Undetermined large	34	3	244	7	82.9	98.8
Undetermined small	28	3	249	8	77.8	98.8

1 **Table 3** Effect of fixatives and buffers on the percentage of each shape observed

			1% osmium tetroxide		
			3mM Hepes	100mM Hepes	100mM PBS
Crescent shapes	*P. acanthamoebae*	3mM Hepes	----	ns	-----
		100mM Hepes		----	-----
		100mM PBS			NA
	W. chondrophila	3mM Hepes	ns	ns	ns
		100mM Hepes		++++	ns
		100mM PBS			ns
	C. sequanensis	3mM Hepes	+++	ns	ns
		100mM Hepes		NA	ns
		100mM PBS			---
	E. lausannensis	3mM Hepes	ns	ns	-
		100mM Hepes		NA	ns
		100mM PBS			ns
Star shapes	*P. acanthamoebae*	3mM Hepes	--	---	ns
		100mM Hepes		ns	+++
		100mM PBS			NA
	W. chondrophila	3mM Hepes	++++	++++	++++
		100mM Hepes		----	++
		100mM PBS			----
	C. sequanensis	3mM Hepes	----	+++	++++
		100mM Hepes		NA	++++
		100mM PBS			---
	E. lausannensis	3mM Hepes	++++	ns	+
		100mM Hepes		NA	+
		100mM PBS			ns
Round shapes	*P. acanthamoebae*	3mM Hepes	++++	+	++++
		100mM Hepes		++++	++++
		100mM PBS			NA
	W. chondrophila	3mM Hepes	ns	++++	-----
		100mM Hepes		----	-----
		100mM PBS			ns
	C. sequanensis	3mM Hepes	----	ns	-----
		100mM Hepes		NA	-----
		100mM PBS			----
	E. lausannensis	3mM Hepes	---	ns	-----
		100mM Hepes		NA	-----
		100mM PBS			ns

NA: not analyzed, ns: not significant, + /- p<0.05, ++/-- p<0.01, +++/--- p<0.001, ++++/---- p<0.0001. Dark grey cells comparison between fixatives (+/- compared to gluataraldehyde). Light grey cells comparison between buffers in 1% osmium tetroxide (+/- compared to buffer in the same column).

Table S1: Osmolarity of fixative and buffer solutions

Osmolarity	(mOsm)	3% glutaraldehyde	1% osmium tetroxide
3mM Hepes	0	954	29
100mM Hepes	96	986	98
100mM PBS	276	1120	250

Samples were measured in duplicates.

Table S2 ImageJ Parameters

Parameter	Definition	Parameter	Definition
Area	Area of the particle	**Feret**	Longest distance between any two points along the selected boundary
Perim.	Perimeter of particle	**MinFeret**	Angle of minimum caliper
ARon Area	AR / area	**AR**	Major axis / minor axis
Width	Width of smallest rectangle enclosing the particle	**Round**	Roundness
Height	Height of smallest rectangle enclosing the particle	**Solidity**	Area / convex area
Major	Major axis of ellipse fitting the particle	**Circ.**	Circularity
Minor	Minor axis of ellipse fitting the particle		

Figures

Fig. 1 Method for quantification of morphological features of bacteria**.** (**a**) Reference shapes were selected by eye and their parameters acquired with ImageJ. Magnification of 7'000x. (**b**) Two parameters do not allow a good discrimination of shapes (**c**) Combination of parameters by the linear discriminant analysis is optimized to separate the different shapes. (**d**) Sensitivity and specificity of LDA predictions based on 100 random training and each complementary test datasets. Color code of figure: crescent: red, star: purple, round: blue, convex polygon: green, undetermined small: brown, undetermined large: black. Undetermined small represent small EBs without defined shape or bacterial debris. Undetermined big represent two EBs too close to allow separate recognition by ImageJ.

Fig. 2 Quantification of bacterial morphological features depending on fixation**.** (**a**) Percentage of bacteria in sample per fixation condition. The proportion of elementary bodies (EBs) detected in each fixation and buffer condition was compared to the total amount of EBs quantified for each bacterium. A lower concentration of bacteria was observed with 1% osmium tetroxide in almost all bacteria. (**b**) For each bacterium the percentage of each shape is represented according to fixation method (compared to the total amount of EBs for each condition and bacteria).

ESM Fig. 1 Pairwise plotting of ImageJ parameters. Values of training set of predetermined shapes (crescent: red, star: purple, round: blue, convex polygon: green, undetermined small: brown, undetermined large: black) are represented.

ESM Fig. 2. Determination of aggregation of bacteria. Electron microscopy images were quantified with ImageJ without size filter and the mean area for each fixatives and buffer condition was determined. Buffers in bold font are with 1% Osmium tetroxide and the regular font with 3% glutaraldehyde.

ESM Fig. 3 Effect of buffer on bacterial morphology. For *E. lausannensis* and *C. sequanensis* the fixation of bacteria with 100mM Hepes buffer in 3% glutaraldehyde caused a marked lysis of the bacteria to a point where there were not enough bacteria left for statistically significant morphotypes analyses. Empty bacteria (phantoms) are highlighted with a white arrowhead. For *P. acanthamoebae* the combination of 3% glutaraldehyde and 100mM PBS also caused a strong lysis of the bacteria. *W. chondrophila* was the most resilient to changes in buffer and fixation, since none of the fixation procedures caused a significant loss of bacteria. For all the bacteria fixed with 1% osmium tetroxide with 100mM PBS a rounder morphology was observed (second row). However, for *P. acanthamoebae* and *W. chondrophila* a partial lysis of the bacteria was observed by the appearance of phantoms. Magnification of 7'000x.

Figure 1
Click here to download Figure: Figure1.eps

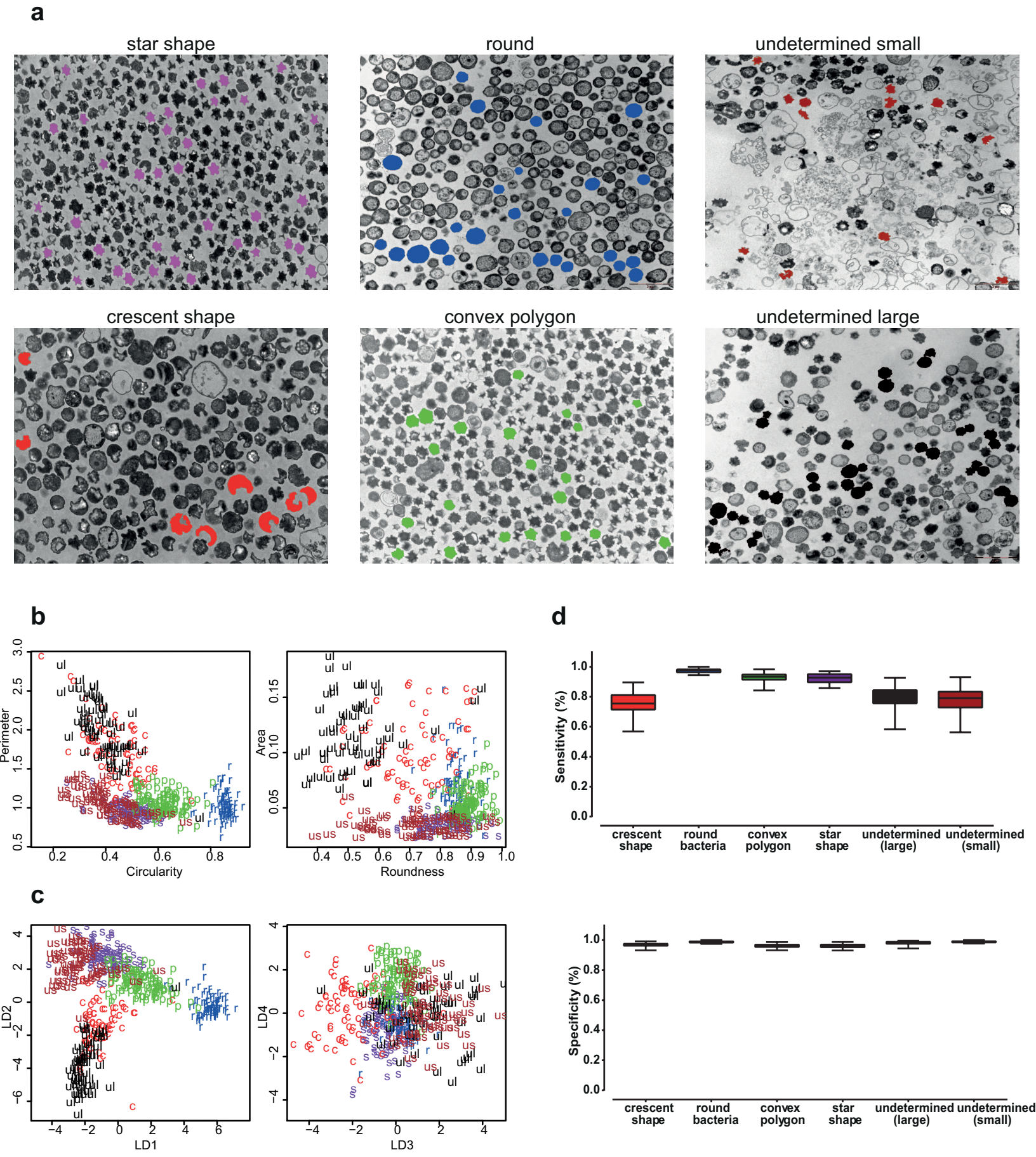
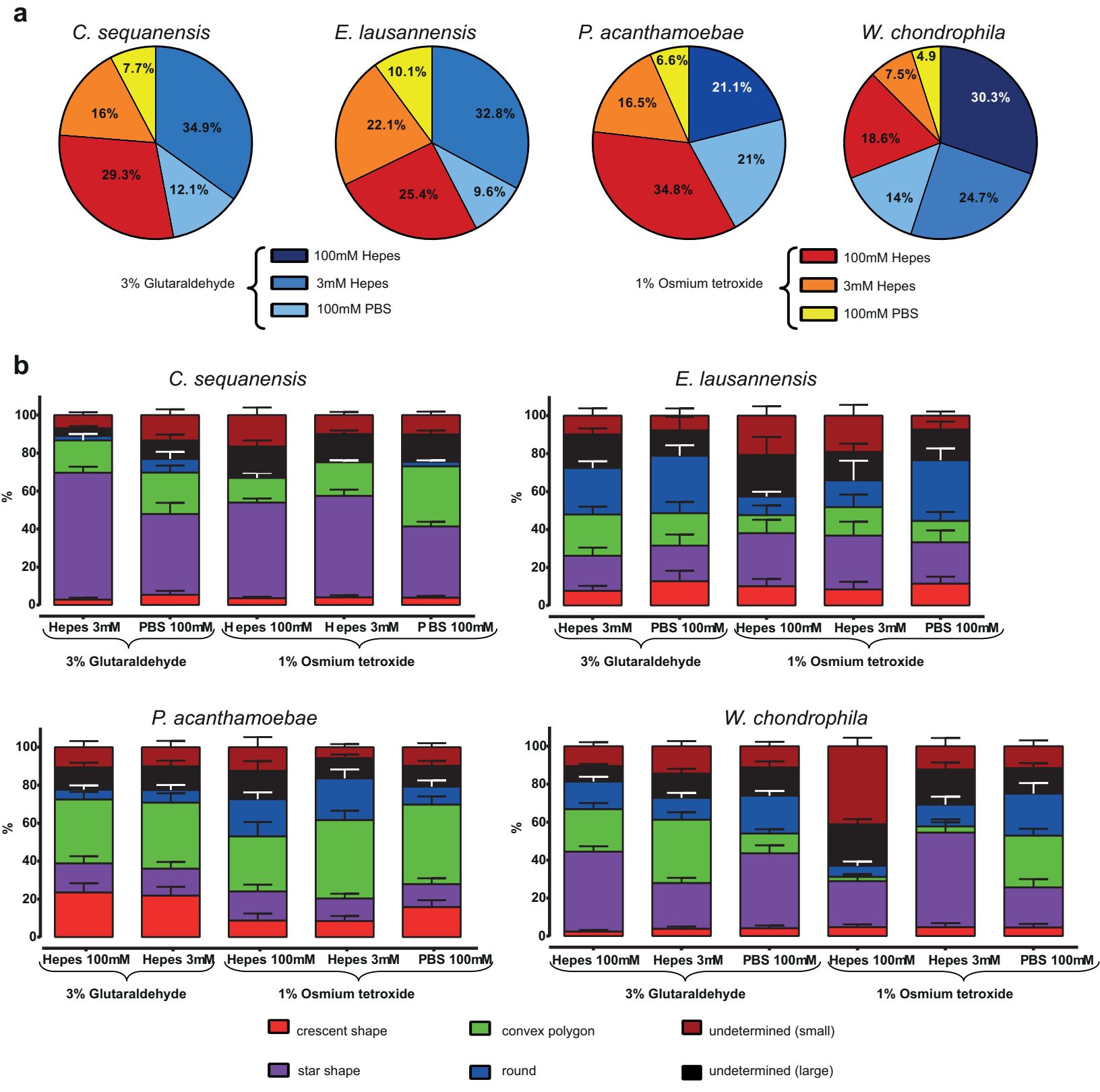


Figure 2
Click here to download Figure: Fig2.eps



SUPPLEMENTARY MATERIAL:

ANTONIE VAN LEEUWENHOEK JOURNAL OF MICROBIOLOGY

Crescent and Star Shapes of Members of the *Chlamydiales* Order: Impact of Fixative Methods

Brigida Rusconi[1*], Julia Lienard[1*], Sébastien Aeby[1], Antony Croxatto, Claire Bertelli[1,2], Gilbert Greub[1]

1 Center for Research on Intracellular Bacteria, Institute of Microbiology, University Hospital Center and University of Lausanne, Lausanne, Switzerland

2 SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland

* These authors contributed equally to this work

Corresponding author

Prof. Gilbert Greub

Institute of Microbiology

Rue du Bugnon 48

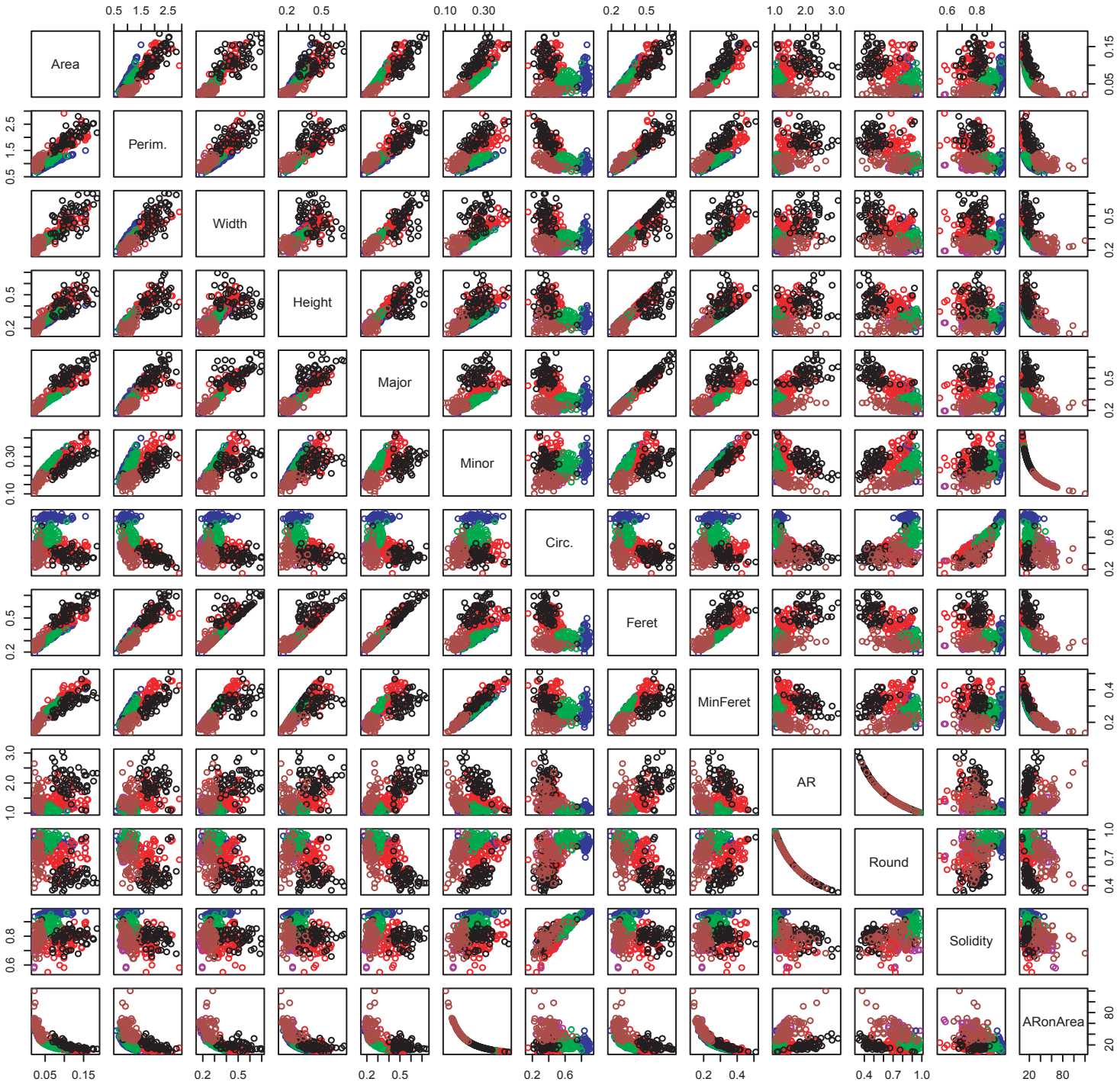
1011 Lausanne

Switzerland

Tel: 0041 21 314 4979

Fax: 0041 21 314 4060

E-mail: gilbert.greub@chuv.ch



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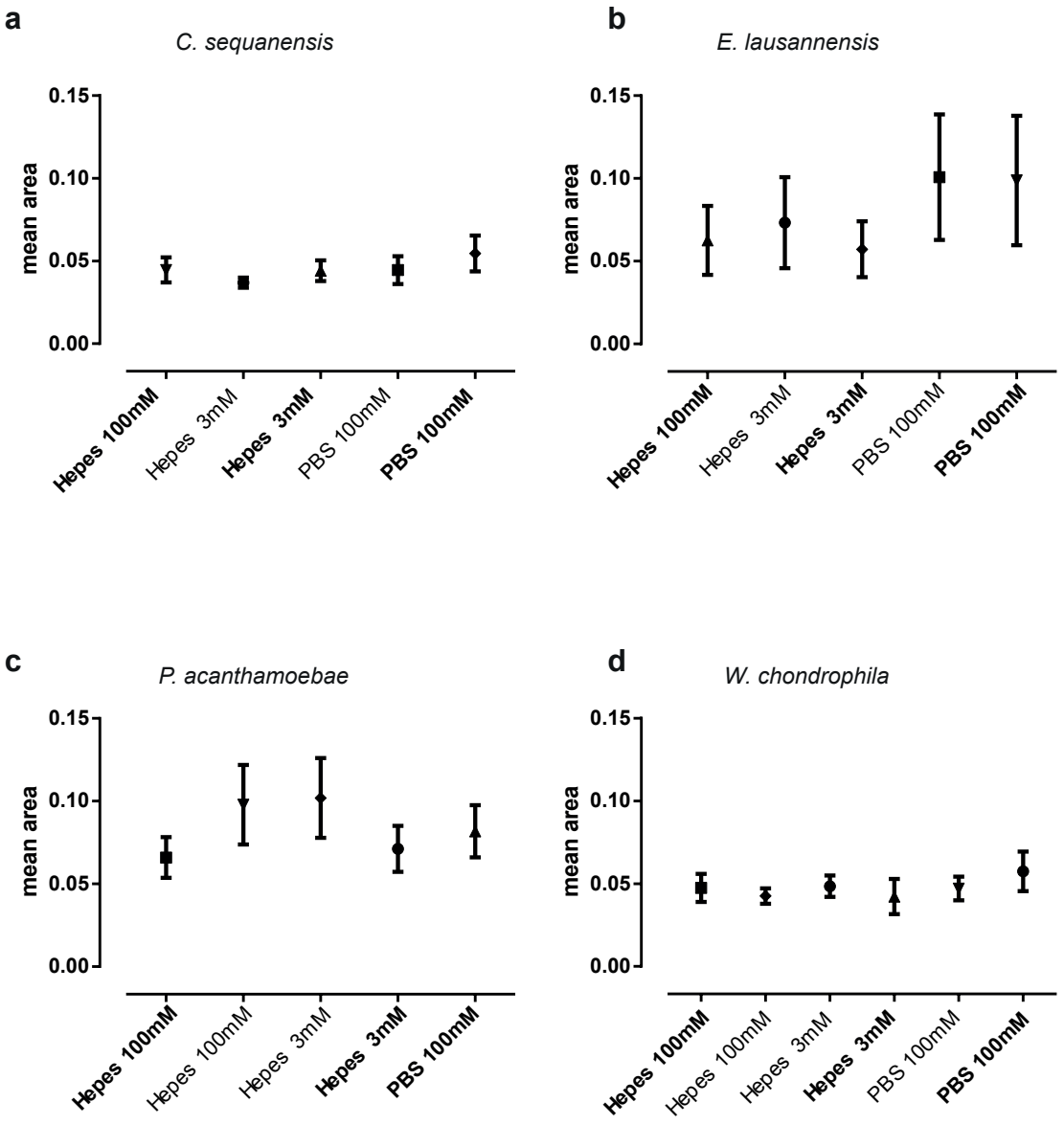

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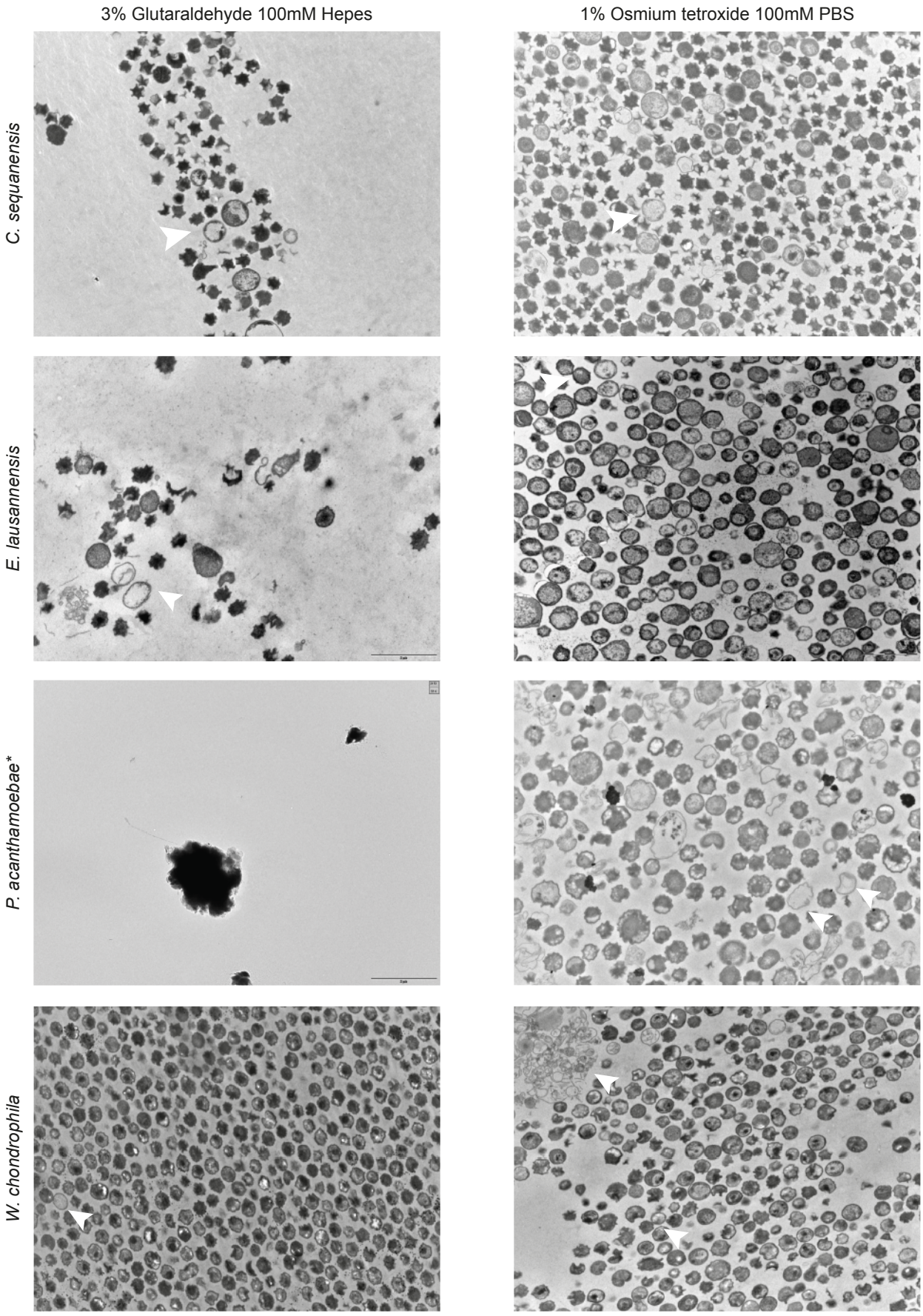

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