nature portfolio

Peer Review File

Extracellular Vesicles of Minimalistic Mollicutes as Mediators of Immune Modulation and Horizontal Gene Transfer

Corresponding Author: Dr THERESA WAGNER

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript Wagner et al., investigate the content and function of extracellular vesicles (EVs) released from minimalistic Mollicutes. To investigate EVs in these small bacterial species are challenging as the EV size almost overlap with the mother bacterium from which they were released. It is also within the limits of the current instrumental resolution. This may question the significance of some of the data presented. Although the authors discuss this in the manuscript it should be more clearly emphasised and additional experiments are required. However, the study is thoroughly performed. The manuscript as a whole is well written and structured, but the methods and results needs better clarification in some instances.

In the introduction bacterial EVs are presented, however, they are only conceptually described as outer membrane vesicles for Gram negative bacteria. It is become apparent that bacterial EV can be produced in various ways as reviewed in Delivery of Toxins and Effectors by Bacterial Membrane Vesicles, by Macion et al MDPI Toxins (2021) or Composition and functions of bacterial membrane vesicles | Nature Reviews Microbiology by Toyofuku et al., 2023. It is suggested that the authors mention this fact in Introduction of the manuscript associated with line 47. This is important particularly considering the results presented in the manuscript e.g. Figure 1 showing a major part of the proteins in the EVs are from cytoplasmic origin and do not differentiate significantly from the originating bacteria as described for other bacterial EV studies.

In the material and methods the author describe that during EV isolation the supernatant was sequentially filtered through 0.45 µm, followed by a 0.22 µm and a 0.1 µm pore filter. The size of EVs is reported to be ~30-300 nm in size which overlap with the Mycobacterium bacterial size. By using the 0.22 and 0.1 um filters the preparations will potentially exclude the larger EV from the preparations which may influence the results (larger EV can have different content compared to smaller EVs). The authors should supply electron microscopy data and size analysis between each filtration step to reveal differences and the EV content between the them.

For the proteomic analysis it is not clear if the EV and mother cell analysis was performed from the same bacterial culture. This should be more clearly described.

The authors have access to NTA analysis that can measure particle size and concentrations so in Line 157 – 158 - do the authors have any what recognition on the concentration/number of EVs present in the nuclease activity? Similarly Line 167: How many EV particles/EV particles per cell were used in the PBMC assays, and in line 222 in the vesiduction experiments? Could they relate the number or concentration to other than ml of bacterial culture?

It is interesting that a immunomodulatory effect of the EVs could be detected. This has also been reported for other bacterial EVs can should be discussed in this context in the discussion.

Line 222. It is more common to write 400 than four hundred µl, and the authors interchange these in the manuscript. Please correct and be consistent.

Lines 262 – 265. In the statement “proteomics showed that 99.8±0.05% were Mycoplasma protein in the bacterial cell samples of Mmc GM12 and 83.2±0.53% were Mycoplasma protein in EV samples of Mmc GM12” it is unclear if the authors means that there were a % of proteins (e.g. 17% in EVs) that were not from Mycoplasma? If this is the case it would be good

to mention what these 17% are. If this is not the case it is suggested to rephrase to clarify what they mean to say. Even though I spent some time going through the supplementary excel file with the proteomics data it was not that easy find this information.

Lines 310 – 313 and Figure 3B describing the nuclease activity in Mmc GM12 in which the authors state that nuclease activity was detected for the linearized plasmid pIVB08EcoRI in presence of salts (10 mM CaCl2 and 10 mM MgCl2) and inhibited by addition of 1 mM EDTA. In Figure 3B results for all samples look very similar and it is difficult to see any difference between the EDTA treated samples and samples treated with 10 mM CaCl2 and 10 mM MgCl2. Can the author provide better explanations.

Reviewer #2

(Remarks to the Author)
This paper provides valuable information for EVs secreted by two veterinary pathogenic mycoplasma species. It contains a large amount of data and text is readable. However, it has some points to be clarified.

Major points
1. This paper should show microscopic images of isolated EVs, including DNA staining, and hopefully electron microscopy. As the isolated EVs do not differ largely from the original bacteria, possibly the isolated particles may be damaged cells.
2. The vesiduction results should be presented in detail with statistics. How did the authors get the “Transfer frequency”? How were the dilution and actucal CFU numbers? Also the authors should show control results using bacterial cells.

Minor points
L24: What is the meaning of “homologous stratin”?
Fig. 3D: Explain the reason why pIVB08EcoRI bands look different between two “EDTA” lanes?
L361: “Of note, the CT values of the small natural plasmid pKMK1 were lower than of the larger oriC-plasmid pIVB08.” Do you mean smaller plasmid is easy to be detected in realtime PCR?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear Editor,

I thank the authors for the their thorough revision of the manuscript and for addressing the reviewers concerns. In my mind the manuscript is acceptable for publication with one minor addition: In material and method section line 135 (new manuscript), please add the manufacturer for Uranyless.

Reviewer #2

(Remarks to the Author)
My concerns have been well addressed.

Dear reviewers,

Thank you for your thorough revision and feedback. Please find our point-by-point answers in blue italics below.

Reviewer #1 In this manuscript Wagner et al., investigate the content and function of extracellular vesicles (EVs) released from minimalistic Mollicutes. To investigate EVs in these small bacterial species are challenging as the EV size almost overlap with the mother bacterium from which they were released. It is also within the limits of the current instrumental resolution. This may question the significance of some of the data presented. Although the authors discuss this in the manuscript it should be more clearly emphasised and additional experiments are required. However, the study is thoroughly performed. The manuscript as a whole is well written and structured, but the methods and results need better clarification in some instances.

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Thank you for this suggestion. We expanded the introduction to acknowledge that bacterial EVs can be produced through various mechanisms (lines 52-56:

> "In Gram-negative bacteria, vesicles can be shed by membrane blebbing from the outer membrane (outer membrane vesicles (OMVs)), the outer-inner membrane (outer-inner membrane vesicles (OIMV)), or explosive cell lysis (explosive membrane vesicle (EMV)), while Gram-positive bacteria shed vesicles from their inner (cytoplasmic) membrane, called membrane vesicles (MVs) [1,3,4]. "

We have also updated the discussion to contextualize our finding that a large proportion of the EV proteins are of cytoplasmic origin, aligning this observation with reports in other bacterial EV studies (line 497-9:

> "Our findings demonstrate that EVs from both *Mmc* and *M. bovis* reflect the overall proteomic profiles of the EV-producing bacterial cell, which is in line with previous descriptions of bacterial vesicles originating from the cytoplasmic membrane [1]."

In the material and methods, the authors describe that during EV isolation the supernatant was sequentially filtered through 0.45 µm, followed by a 0.22 µm and a 0.1 µm pore filter. The size of EVs is reported to be ~30-300 nm in size which overlap with the Mycobacterium bacterial size. By using the 0.22 and 0.1 µm filters the preparations will potentially exclude the larger EV from the preparations which may influence the results (larger EV can have different content compared to smaller EVs). The authors should supply electron microscopy data and size analysis between each filtration step to reveal differences and the EV content between them.

Thank you for this valid point. We have now included images of the bacteria and the EVs (Figure 1).

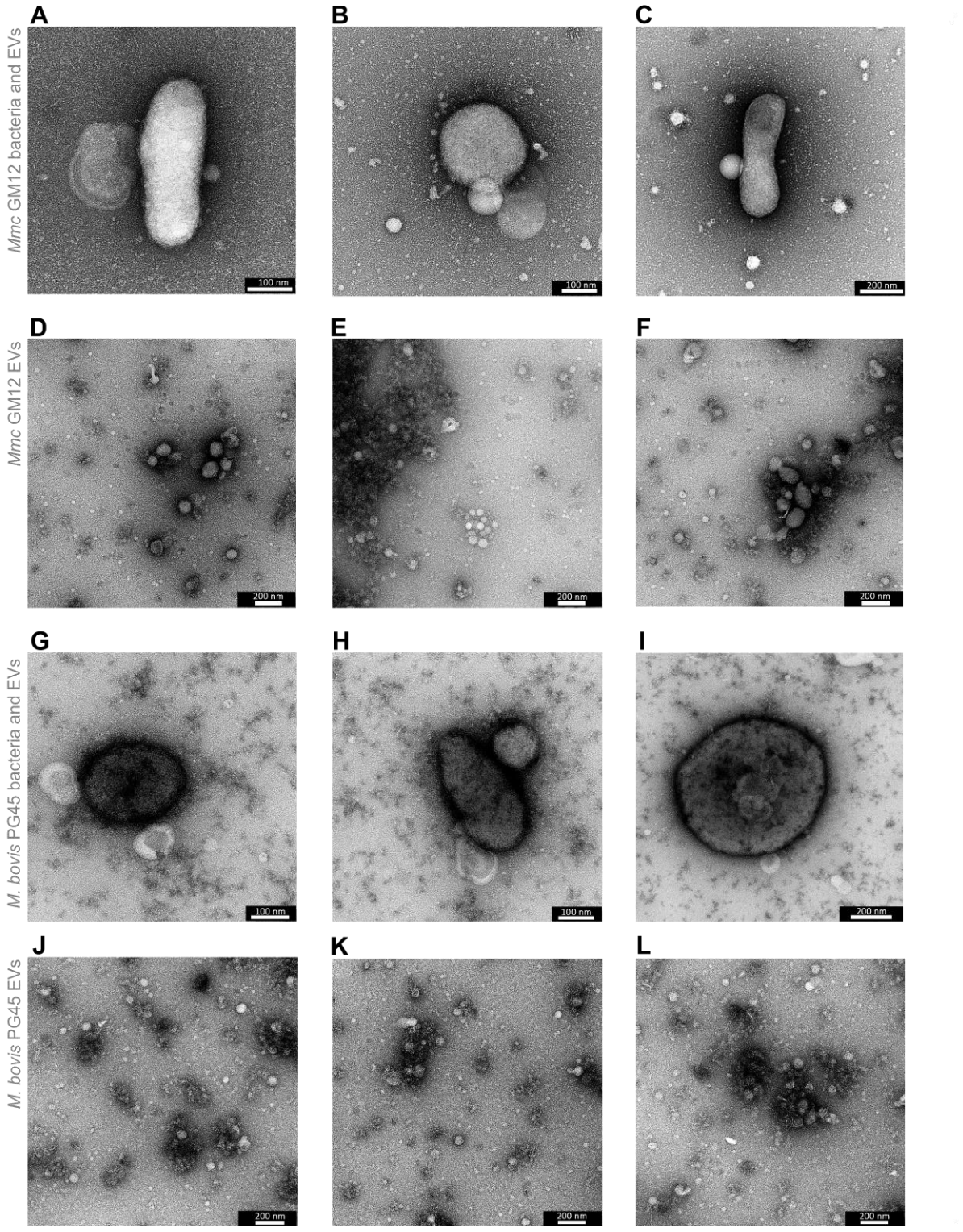


Figure 1: Transmission electron microscopy of bacterial cells and EVs. *Mmc* GM12 cells and EVs (A-C), *Mmc* GM12 EVs after isolation (D-F), *M. bovis* PG45 cells and EVs (G-I) and *M. bovis* PG45 EVs after isolation (J-L) at a magnification of 28,000x (EVs) and 73,000x (cells with EVs)).

We acknowledge that using sequential filtration through 0.45 µm, 0.22 µm, and finally 0.1 µm filters may potentially exclude larger EVs. However, our primary goal was to ensure a bacterial cell-free preparation. Given that mycoplasmas are very small, pleomorphic, and lack a cell wall, they can pass through filters with larger pore sizes. Although we are aware that this procedure may result in the loss

of larger EVs, it is essential to avoid contamination by bacterial cells. Additionally, due to the lack of a cell wall, the morphology of EVs and bacterial cells is quite similar on TEM, which further supports our decision to use only the 0.1 µm bacterial cell-free fraction for subsequent analyses.

The particle size in the EVs after isolation in TEM was 78.5 nm ± 40.05 nm for Mmc GM12 EVs and 61.9 nm ± 25 nm for M. bovis PG45 (n = 100 EVs, average over 10 different TEM images, with STDEV, measured in Image J); with a range from 18.6 nm to 203.1 nm for Mmc GM12 and 18.2 nm to 141.3 for M. bovis PG45 EVs.

We would also like to note that previous studies (Gaurivaud, P. et al. Mycoplasmas are no exception to extracellular vesicles release: Revisiting old concepts. PLoS ONE 13, e0208160 (2018).; Chernov, V. M. et al. Extracellular vesicles derived from Acholeplasma laidlawii PG8. ScientificWorldJournal 11, 1120–1130 (2011).; Chernov, V. M. et al. Extracellular membrane vesicles secreted by mycoplasma Acholeplasma laidlawii PG8 are enriched in virulence proteins. J. Proteomics 110, 117–128 (2014).), have successfully imaged Mycoplasma EVs using similar isolation methods (see figures in the ANNEX extracted from the indicated publications), which supports the validity of our approach.
While we cannot entirely exclude the possibility that some damaged cells might be present in the EV fraction, the centrifugation protocol we used, is well established for enriching EVs in both mycoplasmas and other bacteria. Given that mycoplasmas lack a cell wall and had a common ancestor with Gram-positive Clostridia, the EVs are likely derived from the cytoplasmic membrane, similar to Gram-positive bacteria. We therefore do not expect substantial differences in cargo content compared to the parent cells unless an active sorting mechanism is involved.
Finally, our vesiduction experiments provide functional evidence that we enriched intact EVs: the EVs protect their cargo from degradation, which would not be the case if they were merely cellular debris from damaged cells.

For the proteomic analysis it is not clear if the EV and mother cell analysis was performed from the same bacterial culture. This should be more clearly described.
Thanks, this was added (line 146:

> "For comparison of the proteomic content to bacterial cells, the latter were harvested from 1 ml cultures of the same batch via centrifugation at 14.000 x *g*, washed with an equal volume of PBS, centrifuged at 7.000 x *g* and then stored at -80°C."

The authors have access to NTA analysis that can measure particle size and concentrations so in Line 157 – 158 - do the authors have any what recognition on the concentration/number of EVs present in the nuclease activity?
Thank you for this question. Unfortunately, using NTA to distinguish EVs from the background was not successful. Mycoplasmas are fastidious bacteria that require a highly nutritious and complex growth medium, such as SP5, which contains serum (FBS). Even after reducing FBS from 17% to 2%, the background remained high—likely due to serum-derived aggregates reported elsewhere (see: Lehrich BM, Liang Y, Khosravi P, Federoff HJ, Fiandaca MS. Fetal Bovine Serum-Derived Extracellular Vesicles Persist within Vesicle-Depleted Culture Media. Int J Mol Sci. 2018 Nov 9;19(11):3538. doi: 10.3390/ijms19113538. PMID: 30423996; PMCID: PMC6275013.).
We also considered using nucleic acid quantification as a proxy for vesicle quantification; however, since Mycoplasma also require nucleic acids as nutrients, the SP5 medium inherently contains nucleic acids. Thus, measurements of nucleic acids in the EV sample could derive from both the EVs and the medium. Only after DNase treatment and using specific qPCR targets can we confidently assign nucleic

acids to EV content.
Given these challenges, we chose an indirect approach. We estimated EV content by combining protein quantification with proteomic data—identifying a defined percentage of proteins as Mycoplasma-derived. Moreover, we found that relating EV numbers to the number of bacterial cells in the culture provided the most consistent and reproducible measure, which should facilitate replication by others.

Similarly Line 167: How many EV particles/EV particles per cell were used in the PBMC assays, and in line 222 in the vesiduction experiments? Could they relate the number or concentration to other than ml of bacterial culture?
As explained in detail above, we chose to relate the EVs to the bacterial cell number.

It is interesting that a immunomodulatory effect of the EVs could be detected. This has also been reported for other bacterial EVs can should be discussed in this context in the discussion.
This has now been added to the discussion (line 548-9:

> "The immunomodulatory capacity of bacterial EVs has been recognized for other bacteria, too [68,69], which is in line with our findings"

Line 222. It is more common to write 400 than four hundred µl, and the authors interchange these in the manuscript. Please correct and be consistent.
This has been corrected.

Lines 262 – 265. In the statement "proteomics showed that 99.8±0.05% were Mycoplasma protein in the bacterial cell samples of Mmc GM12 and 83.2±0.53% were Mycoplasma protein in EV samples of Mmc GM12" it is unclear if the authors means that there were a % of proteins (e.g. 17% in EVs) that were not from Mycoplasma? If this is the case it would be good to mention what these 17% are. If this is not the case it is suggested to rephrase to clarify what they mean to say. Even though I spent some time going through the supplementary excel file with the proteomics data it was not that easy find this information.
Thanks for pointing this out. For better clarity we now added a column in the table, indicating for each protein whether it is a Mycoplasma protein or not ("PG.Organisms"). We also updated the materials and methods section to include more detail on the sample preparation and analysis.
As explained in detail above, mycoplasmas require rich, serum-containing medium for growth and the non-Mycoplasma protein originates from the growth medium.

Lines 310 – 313 and Figure 3B describing the nuclease activity in Mmc GM12 in which the authors state that nuclease activity was detected for the linearized plasmid pIVB08EcoRI in presence of salts (10 mM CaCl2 and 10 mM MgCl2) and inhibited by addition of 1 mM EDTA. In Figure 3B results for all samples look very similar and it is difficult to see any difference between the EDTA treated samples and samples treated with 10 mM CaCl2 and 10 mM MgCl2. Can the author provide better explanations.
Thank you for pointing this out. The label in figure 3B was off in the 3rd to last lane and this has been corrected now. The reason why GM12 samples look similar, is probably that the nuclease activity is not as high as in PG45. Also, for GM12 we only detected exo- and no endonuclease activity, as described. 10 mM $CaCl_2$ or 10 mM $MgCl_2$ alone were not sufficient for nuclease activity and only when both 10 mM $CaCl_2$ plus 10 mM $MgCl_2$ were added activity was detected. The reason why we included one PG45 sample on the GM12 gel, is that nuclease activity for PG45 had been previously reported (Gelgie, A. E.

et al. Mycoplasma bovis 5’-nucleotidase is a virulence factor conferring mammary fitness in bovine mastitis. PLOS Pathog. 20, e1012628 (2024)., Sharma, S., Tivendale, K. A., Markham, P. F. & Browning, G. F. Disruption of the Membrane Nuclease Gene (MBOVPG45_0215) of Mycoplasma bovis Greatly Reduces Cellular Nuclease Activity. J. Bacteriol. 197, 1549–1558 (2015) and we thus used PG45 as a positive control.

Reviewer #2

This paper provides valuable information for EVs secreted by two veterinary pathogenic mycoplasma species. It contains a large amount of data and text is readable. However, it has some points to be clarified.

Major points

1. This paper should show microscopic images of isolated EVs, including DNA staining, and hopefully electron microscopy. As the isolated EVs do not differ largely from the original bacteria, possibly the isolated particles may be damaged cells.

Thank you for pointing this out. We have now included TEM images of the bacteria and the EVs (Figure 1). Please see the extensive answer give to reviewer 1 above.

2. The vesiduction results should be presented in detail with statistics. How did the authors get the "Transfer frequency"? How were the dilution and actual CFU numbers? Also, the authors should show control results using bacterial cells.

Thank you for your comment. We have now expanded the vesiduction section and included additional controls, including incubation with plasmid alone and bacteria-to-bacteria transfer (lines 269-472:

> The transfer frequency was calculated as the number of transformants per recipient CFU. Recipient CFUs were counted on non-selective plates, while transformants were counted on SP5 plates supplemented with tetracycline (5 µg/ml for *Mmc* GM12 and *Macp*; 15 µg/ml for *M. leachii*).*,*

287-292: As control, *Mcap* ΔRE and *M. leachii* were co-incubated for 90 min with 10 µg pIVB08 following protocol B and plated on selective tetracycline plates. Bacteria-to-bacteria control was performed using cells of *Mmc* GM12 pIVB08 and *Mcap* ΔRE. The two strains were co-incubated for 90 min at a ratio of 1:1, with 10^{11} total CFUs, following protocol B and plated on double selection plates with tetracycline (selecting for pIVB08) and puromycin (selecting for *Mcap* ΔRE).

460-462: When *Mcap* ΔRE or *M. leachii* were co-incubated as in protocol B with the pIVB08 plasmid alone, no transformants were detected. Similarly, bacterial cells of *Mmc* GM12 pIVB08 and *Mcap* ΔRE co-incubated did not result in transformants. *, Table 1).*

We also repeated homologous vesiduction to Mmc GM12 and heterologous vesiduction to Mcap ΔRE, focusing on key vesiduction pairs and their controls (Table 1).

The transfer frequency was calculated as the number of transformants (vesiculants) per recipient CFU. Recipient CFUs were counted on non-selective plates, while vesiculants were counted on SP5 plates supplemented with tetracycline. This was added to the methods now (see lines 238-241).

During protocol optimization, we aimed for colony counts between 10 and 100 transformants per plate. The actual counted transformant CFU values ranged from approximately 20 to 50 CFUs, counted using a stereo microscope. When PEG was added to the fusion buffer (protocol C), a higher transformant CFU count was expected, so we used fewer recipient cells (10^8), leading to a final count of 196 CFUs per plate. This is a high number, but since the Mcap colonies were small, they could still be countable and not growing into each other.

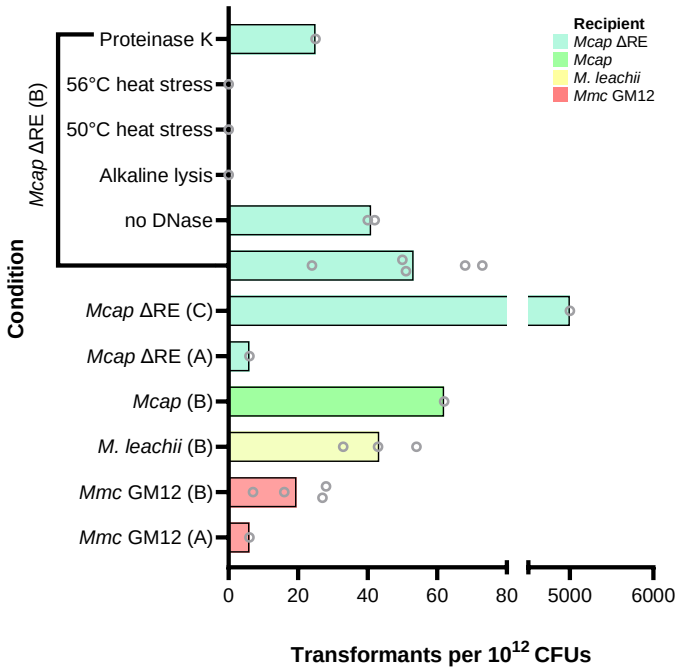
Previous papers describing vesiduction in Gram-negative bacteria report data in a similar way (see "TABLE 3 Gene transfer frequencies and antibiotic susceptibility profiles of vesiculants of A. baylyi and E. coli" in Fulsundar, S. et al. Gene Transfer Potential of Outer Membrane Vesicles of Acinetobacter baylyi and Effects of Stress on Vesiculation. Appl. Environ. Microbiol. 80, 3469–3483 (2014); and also, Tran, F. & Boedicker, J. Q. Plasmid Characteristics Modulate the Propensity of Gene Exchange in Bacterial Vesicles. J. Bacteriol. 201, 10.1128/jb.00430-18 (2019).)

We believe that presenting these data in Table 1 provides a more informative and comprehensive overview. For review, we have also included an additional figure below, visualizing the vesiduction data with statistical analysis.

Table 1 Plasmid pIVB08 transfer frequencies in homo- and heterologous vesiduction.

Donor pIVB08	Recipient cell	Vesiduction protocol*	Mean transfer frequency#
EV_{pIVB08}	*Mmc* GM12	A	6 per 10^{11}CFU $^{(n=1)}$
EV_{pIVB08}	*Mmc* GM12	B	19 per 10^{11}CFU $^{(n=4)}$
EV_{pIVB08}	*M. leachii*	B	43 per 10^{11}CFU $^{(n=3)}$
EV_{pIVB08}	*Mcap*	B	62 per 10^{11}CFU $^{(n=1)}$
EV_{pIVB08}	*Mcap* ΔRE	A	6 per 10^{11}CFU $^{(n=1)}$
EV_{pIVB08}	*Mcap* ΔRE	B	53 per 10^{11}CFU $^{(n=5)}$
EV_{pIVB08}	*Mcap* ΔRE	C	1 per 5x10^{7}CFU $^{(n=1)}$
EV_{pIVB08} (Alkaline lysis)	*Mcap* ΔRE	B	0 $^{(n=1)}$
EV_{pIVB08} (50°C heat stress)	*Mcap* ΔRE	B	0 $^{(n=1)}$
EV_{pIVB08} (56°C heat stress)	*Mcap* ΔRE	B	0 $^{(n=1)}$
EV_{pIVB08} (Proteinase K)	*Mcap* ΔRE	B	2.5 per 10^{10}CFU $^{(n=1)}$
Mmc GM12 pIVB08 bacteria	*Mcap* ΔRE	B	0 $^{(n=3)}$
pIVB08 (naked plasmid)	*Mcap* ΔRE	B	0 $^{(n=3)}$
pIVB08 (naked plasmid)	*M. leachii*	B	0 $^{(n=3)}$

*A co-incubation, B standard protocol, C standard protocol with 10% PEG_{6000}, # n = number of replicates



Additional figure for review: **Plasmid pIVB08 transfer frequencies in homo- and heterologous vesiduction**. Transformants are given per 10^{12} CFUs of recipient cells under the different conditions, using the protocols co-incubation(A), standard protocol (B), or standard protocol with 10% PEG_{6000}. Bars show the median of replicates and individual replicates are shown as circles

Minor points

L24: What is the meaning of “homologous strain”?

*We meant the strain producing EVs, the term has been changed throughout the manuscript for easier readability (lines 30, 64, 498: “*EV-producing bacteria*”).*

Fig. 3D: Explain the reason why pIVB08EcoRI bands look different between two “EDTA” lanes?

Thank you for pointing this out. The label in figure 3B was off in the 3rd to last lane and this has been corrected now.

L361: “Of note, the CT values of the small natural plasmid pKMK1 were lower than of the larger oriC-plasmid pIVB08.” Do you mean smaller plasmid is easy to be detected in real time PCR?

The lower CT values indicate that the smaller natural plasmid pKMK1 (1.8 kb) is present in higher numbers in the EVs compared to the larger oriC-plasmid pIVB08 (6 kb). Importantly, the qPCR assay is designed with comparable amplicon sizes for both plasmids (see Table S1), ensuring that the detection sensitivity is not affected by the overall plasmid size.

ANNEX

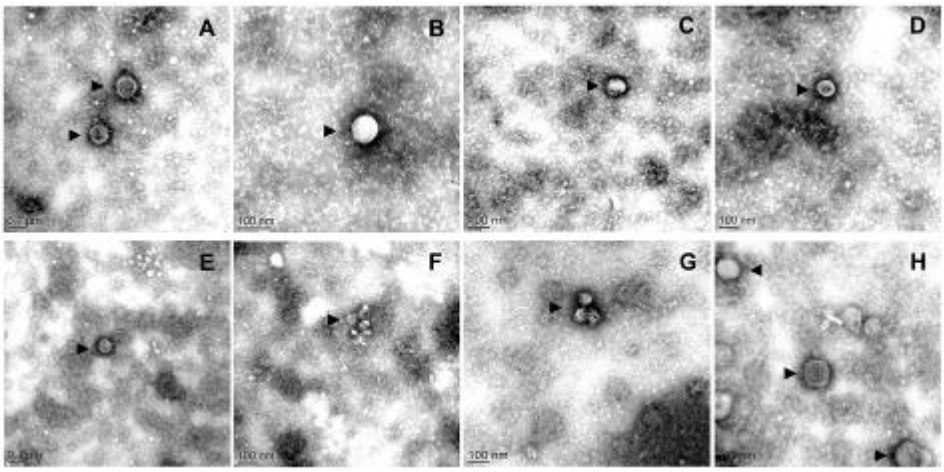


Fig 2. Electron micrographs of negatively-stained EV purified from A. laidlaiwii PG8T (A), M. mycoides subsp. mycoides Afadé (B), M. mycoides subsp. capri PG3T (C), M. capricolum subsp. capricolum L15937 (D), M. agalactiae 5632 (E), M. agalactiae L14628 (F), M. bovis L15762 (G) and M. fermentans PG18T (H). Examples of EV are indicated by black arrowheads. Taken from Gaurivaud, P. et al. Mycoplasmas are no exception to extracellular vesicles release: Revisiting old concepts. PLoS ONE 13, e0208160 (2018).

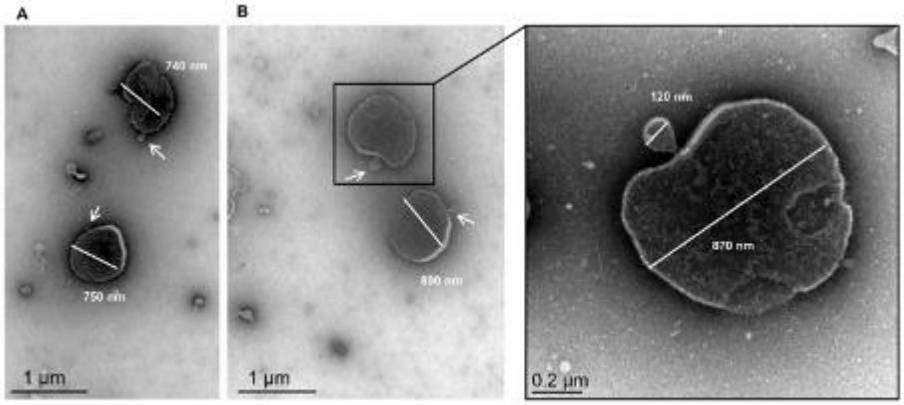


Fig 4. Transmission electron micrographs (A, B) of *M. mycoides* subsp. *mycoides* (strain Afadé) cells showing vesicle-like structures budding from the surface of the mycoplasma cells. A zoom of micrograph B is shown in the right panel. Diameters were estimated using image].

Taken from Gaurivaud, P. et al. Mycoplasmas are no exception to extracellular vesicles release: Revisiting old concepts. PLoS ONE 13, e0208160 (2018).

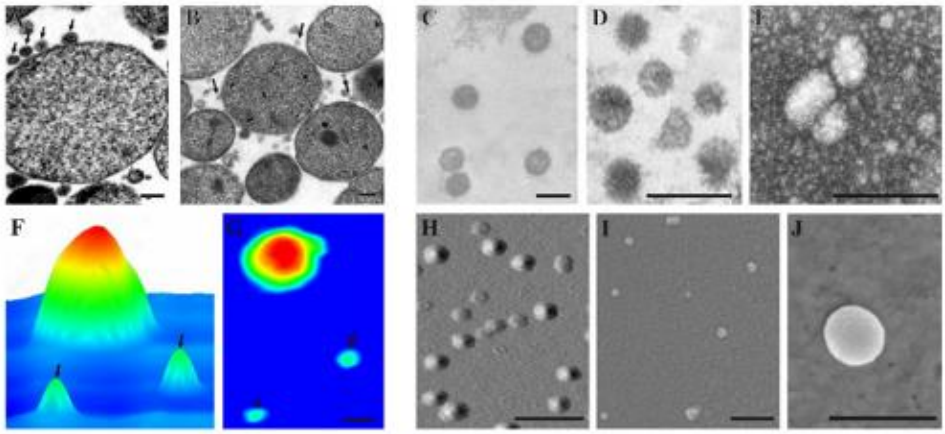


Fig. 1 – TEM (A–E), AFM (F–H) and SEM (I, J) of cells and EVs from *A. laidlawii* PG8. EVs of mycoplasma are indicated using arrows. Bar = 200 nm.

Taken from Chernov, V. M. *et al.* Extracellular membrane vesicles secreted by mycoplasma Acholeplasma laidlawii PG8 are enriched in virulence proteins. *J. Proteomics* **110**, 117–128 (2014)

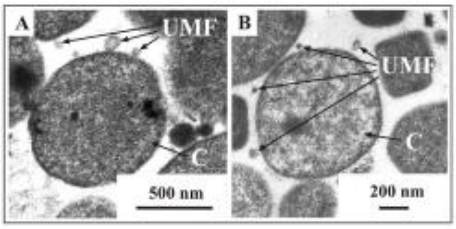


FIGURE 3. Cells and ultramicroforms of *A. laidlawii* PG8. C = "typical" mycoplasma cells; UMF = ultramicroforms.

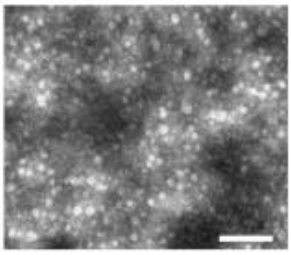


FIGURE 4. Negative-staining TEM of purified ultramicroforms of *A.laidlawii* PG8 after buoyant density-gradient centrifugation. Bar corresponds to 100 nm.

Taken from Chernov, V. M. *et al.* Extracellular vesicles derived from Acholeplasma laidlawii PG8. *ScientificWorldJournal* **11**, 1120–1130 (2011).