OptiPrep™ Mini-Review MS16

Bacterial and fungal extracellular vesicles

1. Introduction

It is widely recognized that mammalian cells, bacteria and fungi release extracellular vesicles into the surrounding medium; these vesicles are involved in communication between cells and the delivery of biologically and clinically important molecules to other cells. With regard to bacteria and fungi, the term "extracellular vesicles" (EVs) covers the outer membrane vesicles (OMVs) produced by Gram-negative bacteria and the membrane vesicles (MVs) produced by Gram-positive bacteria and other organisms such as fungi. In all cases; EVs are distinct from the intracellular vesicles present in the cytoplasm. The OMVs from Gram-negative bacteria in particular are widely researched and have been shown to be important in the transfer of virulence factors and the initiation of immune and inflammatory responses in host cells.

- **Section 2** briefly reviews the current OptiPrep™-based methodology; this is described in detail in Application Sheet S60. It has its own short reference list
- The principal aim of this Mini-Review however is to provide a bibliography of all the published papers that have reported the use of an iodixanol gradient.
  - **Section 3a is devoted to Gram –ve bacteria**
  - **Section 3b to Gram +ve bacteria and mycobacteria**
  - **Section 3c to mycoplasmas**
  - **Section 3d to fungi**
  - **Section 3e to the relatively new Biofilm structures**
  - **Section 3f contains references to methodological reviews**
  - **All references are listed alphabetically according to first author**

Related research areas that have reported the use of gradients prepared from OptiPrep™ are:

- The analysis of the microvesicles that are expressed from the surface of mammalian cells is covered in OptiPrep™ Mini-Review MS17 and Application Sheet S61
- The control and organization of membrane trafficking within mammalian cells that permits the movement of vesicles to, and ultimately their fusion with, the plasma membrane or a specific plasma membrane domain is covered in Optiprep™ Mini-Review MS10 and OptiPrep™ Application Sheet S45
- These other Mini-Reviews or OptiPrep™ Application Sheets can be accessed from the OptiPrep™ flash drive or from the following website: www.axis-shield-density-gradient-media.com, click on "Mini-Reviews" or "Methodology". On the flash drive return to "Mini-Reviews" or "Application Sheets".

2. Methodological summary

Various forms of pre-gradient processing are employed, during which intact bacteria and aggregated material in the culture medium are mostly removed and the EVs concentrated. This is covered in much greater detail in Application Sheet S60

The first step is clarification of the bacterial broth to remove intact cells by centrifugation; the time and *g*-force used varies widely and reflects the size of the organism. Generally rcfs of approx. 10,000 *g* are used for 10-20 min, but there are examples both of lower and higher *g*-forces so that the range spans 4,000-12,000 *g*. Occasionally this first step is carried out in two stages in which the broth is centrifuged for 30 min at 5,000 *g* and then the supernatant at 7,500 *g*. This strategy may minimize the entrapment (and consequent loss) of vesicles into the pellet by the rapidly sedimenting much larger bacteria. Fungal cells appear to sediment satisfactorily at lower speeds such as 2,500 *g* for 10 min. **Commonly the second step is volume reduction** since ultimately the vesicles will be sedimented in a fixed-angle ultracentrifuge rotor, prior to gradient purification. Large volumes of clarified medium

can pose a problem for this final pre-gradient step. The Beckman 45Ti for example which accommodates 6x94 ml tubes has a maximum *g*-force of 235,000 *g*, but the vesicles at the top of the sample experience initially only 80,000 *g*. Tangential filtration devices (e.g. 70-100 kDa cut-off) are popular or centrifugal filters are popular for this volume reduction step.

Residual bacteria in the broth may then be removed by vacuum filtration through either a 0.45 or 0.22 μm filter or both pore size filters sequentially, before the EVs are sedimented in a fixed-angle rotor; the conditions vary quite widely, from approx. 40,000 *g* for 1 h to 140-150,000 *g* for 2-3 h..

Ammonium sulphate precipitation of OMVs from the clarified broth of Gram-ve bacteria has been used a few cases. The precipitation process may only require about half an hour at 4°C but can be as long as overnight. Moreover the resuspended pellet requires dialysis overnight prior to further processing.

Iodixanol gradient purification of the EVs, regardless of the pre-gradient technology, involves adjustment of the suspension to a density of approx. 1.241 g/ml (sometimes higher 1.267 g/ml or lower 1.215 g/ml).for subsequent flotation through a discontinuous iodixanol gradient; During centrifugation at 100-200,000 *g* for 12-16 h the gradient will become more or less continuous; gradients run for shorter times (e.g. 2 h) will retain some of their discontinuous nature.

The density of MVs in iodixanol gradients is generally >1.11 g/ml, many banding between 1.13 and 1.15 g/ml; although occasionally densities of as a high as 1.20 g/ml have been observed. There is also evidence of heterogeneity amongst EV populations from a single bacterial type.

The first published paper to describe the use of OptiPrep™, of which we have a record, was by Horstman and Kuehn, published in 2000. It documented the purification of OMVs from enterotoxin-containing *Escherichia coli*; the OMVs were shown to contain the pathogenic enterotoxin, lipids and specific proteins characteristic of the outer membrane and proteins from the periplasmic space, but no markers of the cytosol or inner membrane (**see Section 3a - *Escherichia coli***).

3. Bibliography of publications reporting analytical studies on iodixanol-purified OMVs and MVs (references are sorted alphabetically according to first author)

3a. Gram-negative bacteria

Aggregatibacter actinomycetemcomitans

Rompikuntal, P.K., Thay, B., Khan, M.K., Alanko, J., Penttinen, A-M., Asikainen, S., Wai, S.N. and Oscarsson, J. (2012) *Perinuclear localization of internalized outer membrane vesicles carrying active cytolethal distending toxin from Aggregatibacter actinomycetemcomitans* Infect. Immun., **80**, 31-42

Burkholderia pseudomallei

Nieves, W., Heang, J., Asakrah, S., Höner zu Bentrup, K., Roy, C.J. and Morici, L.A. (2010) *Immuno-specific responses to bacterial elongation factor Tu during Burkholderia infection and immunization* PloS One **5**: e14361

Borrelia burgdorferi

Coleman, J.L., Crowley, J.T., Toledo, A.M. and Benach, G.L. (2013) *The HtrA protease of Borrelia burgdorferi degrades outer membrane protein BmpD and chemotaxis phosphatase CheX* Mol. Microbiol., **88**, 619–633

Crowley, J.T., Toledo, A.M., LaRocca, T.J., Coleman, J.L., London, E. and Benach, J.L. (2013) *Lipid exchange between Borrelia burgdorferi and host cells* PLoS Pathog., **9**: e1003109

Toledo, A., Coleman, J.L., Kuhlow, C.J., Crowley, J.T. and Benach, J.L. (2012) *The enolase of Borrelia burgdorferi is a plasminogen receptor released in outer membrane vesicles* Infect. Immun., **80**, 359-368

Campylobacter jejuni

Jang, K-S., Sweredoski, M.J., Graham, R.L.J., Hess, S. and Clemons Jr., W.M. (2014) *Comprehensive proteomic profiling of outer membrane vesicles from Campylobacter jejuni* J. Proteom., **98**, 90-98

Escherichia coli

Balsalobre, C., Silvan, J.M., Berglund, S., Mizunoe, Y., Uhlin, B.E. and Wai, S.N. (2006) *Release of the type I secreted α-haemolysin via outer membrane vesicles from Escherichia coli* Mol. Microbiol., **59**, 99-112

Bielaszewska, M., Ruter, C., Kunsmann, L., Greune, L., Bauwens, A., Zhang, W., Kuczius, T., Kim, K.S., Mellmann, A., Schmidt, M.A. and Karch, H. (2013) *Enterohemorrhagic Escherichia coli hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis* PloS Pathog., **9**: e1003797

Blenkiron, C., Simonov, D., Muthukaruppan, A., Tsai, P., Dauros, P., Green, S., Hong, J., Print, C.G., Swift, S. and Phillips, A.R. (2016) *Uropathogenic Escherichia coli releases extracellular vesicles that are associated with RNA* PLoS One, **11**, e0160440

Chen, L., Valentine, J.L., Huang, C-Jr., Endicott, C.E., Moeller, T.D., Rasmussen, J.A., Fletcher, J.R., Boll, J.M. et al (2016) *Outer membrane vesicles displaying engineered glycotopes elicit protective antibodies* Proc. Natl. Acad. Sci. USA, **113**, E3609–E3618

Daleke-Schermerhorn, M.H., Felix, T., Soprova, Z., ten Hagen-Jongman, C.M., Vikström, D., Majlessi, L., Beskers, J., Follmann, F., de Punder, K., van der Wel, N.N. et al (2014) *Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach* Appl, Environ. Microbiol., **80**, 5854–5865

Davis, J.M., Carvalho, H.M., Rasmussen, S.B. and O'Brien, A.D. (2006) *Cytotoxic necrotizing factor type 1 delivered by outer membrane vesicles of uropathogenic Escherichia coli attenuates polymorphonuclear leukocyte antimicrobial activity and chemotaxis* Infect. Immun., 74, 4401-4408

Ficurilli, M., Liu, C., Riviello, C., Pozo, M.J. and Meers, P.R. (2015) *Delivery of liposomal contents to outer membrane vesicles from Gram negative bacteria* Biophys. J. **108 (Suppl.1)**, 408a

Ghosal, A., Upadhyaya, B.B., Fritz, J.V., Heintz-Buschart, A., Desai, M.S., Yusuf, D., Huang, D. et al (2015) *The extracellular RNA complement of Escherichia coli* Microbiol.Open, **4,** 252–266

Horstman, A.L. and Kuehn, M.J. (2000) *Enterotoxigenic Escherichia coli secretes active heat-labile enterotoxin via outer membrane vesicles* J. Biol. Chem., 275, 12489-12496

Kesty, N.C. and Kuehn, M.J. (2004) *Incorporation of heterologous outer membrane and periplasmic proteins into Escherichia coli outer membrane vesicles* J. Biol. Chem., **279**, 2069-2076

Kim, J-Y., Doody, A.M., Chen, D.J., Cremona, G.H., Shuler, M.L., Putnam, D. and DeLisa, M.P. (2008) *Engineered bacterial outer membrane vesicles with enhances functionality* J. Mol. Biol., **380**, 51-66

Kim, O.Y., Choi, S.J., Jang, S.C., Park, K-S., Kim, S.R., Choi, J.P., Lim, J.H., Lee, S-W. et al (2015) *Bacterial protoplast-derived nanovesicles as vaccine delivery system against bacterial infection* Nano Lett. **15**, 266−274

Kunsmann, L., Rüter, C., Bauwens, A., Greune, L., Glüder, M., Kemper, B., Fruth, A., Wai, S.N., He, X., Lloubes, R. et al (2015) *Virulence from vesicles: Novel mechanisms of host cell injury by Escherichia coli O104:H4 outbreak strain* Sci. Rep., **5**: 13252

McBroom, A.J., Johnson, A.P., Vemulapalli, S. and Kuehn, M.J. (2006) *Outer membrane vesicle production by Escherichia coli is independent of membrane instability* J. Bacteriol., **188**, 5385-5392

McBroom, A.J. and Kuehn, M.J. (2007) *Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response* Mol. Microbiol., **63**, 545-558

Park, M., Sun, Q., Liu, F., DeLisa, M.P. and Chen, W. (2014) *Positional assembly of enzymes on bacterial outer membrane vesicles for cascade reactions* PloS One, **9**: e97103

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O., Kiug, L., Gadermaier, B. et al (2016) *A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria* Nat. Comm., **7**: 10515

Roy, K., Hamilton, D.J., Munson, G.P. and Fleckenstein, J.M. (2011) *Outer membrane vesicles induce Immune responses to virulence proteins and protect against colonization by enterotoxigenic Escherichia coli* Clin. Vaccine Immunol., **18**, 1803–1808

Valentine, J.L., Chen, L., Perregaux, E.C., Weyant, K.B., Rosenthal, J.A., Heiss, C., Azadi, P., Fisher, A.C., Putnam, D. et al (2016) *Immunization with outer membrane vesicles displaying designer glycotopes yields class-switched, glycan-specific antibodies* Cell Chem. Biol., **23**, 655–665

Flavobacterium columnare

Laanto, E., Penttinen, R.K., Bamford, J.K.H. and Sundberg, L-R. (2014) *Comparing the different morphotypes of a fish pathogen - implications for key virulence factors in Flavobacterium columnare* BMC Microbiol., **14**:170

Francisella novicida

Chen, L., Valentine, J.L., Huang, C-Jr., Endicott, C.E., Moeller, T.D., Rasmussen, J.A., Fletcher, J.R., Boll, J.M. et al (2016) *Outer membrane vesicles displaying engineered glycotopes elicit protective antibodies* Proc. Natl. Acad. Sci. USA, **113**, E3609–E3618

McCaig, W.D., Koller, A. and Thanassi, D.G. (2013) *Production of outer membrane vesicles and outer membrane tubes by Francisella novicida* J. Bacteriol., **195**, 1120-1132

Francisella tularensis
Chen, L., Valentine, J.L., Huang, C-Jr., Endicott, C.E., Moeller, T.D., Rasmussen, J.A., Fletcher, J.R., Boll, J.M. et al (2016) *Outer membrane vesicles displaying engineered glycotopes elicit protective antibodies* Proc. Natl. Acad. Sci. USA, **113**, E3609–E3618

Haemophilus influenzi
Roier, S., Blume, T., Klug, L., Wagner, G.E., Elhenawy, W., Zangger, K., Prassl, R., Reidl, J., Daum, G., Feldman, M.F. and Schild, S. (2015) *A basis for vaccine development: comparative characterization of Haemophilus influenzae outer membrane vesicles* Int. J. Med. Microbiol., **305**, 298–309
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Sharpe, S.W., Kuehn, M.J. and Mason, K.M. (2011) *Elicitation of epithelial cell-derived immune effectors by outer membrane vesicles of non-typeable haemophilus influenzae* Infect. Immun., **79**, 4361-4369

Haemophilus parasuis
McCaig, W.D., Loving, C.L., Hughes, H.R. and Brockmeier, S.L. (2016) *Characterization and vaccine potential of outer membrane vesicles produced by Haemophilus parasuis* PLoS One **11**: e0149132

Legionella pneumophila
Fernandez-Moreira, E., Helbig, J.H. and Swanson, M.S. (2006) *Membrane vesicles shed by Legionella pneumophila inhibit fusion of phagosomes with lysosomes* Infect. Immun., **74**, 3285-3295

Marinobacter guineae – see ***Shewanella livingstonensis***

***Myobacterium tuberculosis* – see Section 3b**

Neisseria gonorrhoeae
Pérez-Cruz, C., Delgado, L., López-Iglesias, C. and Mercade, E. (2015) *Outer-inner membrane vesicles naturally secreted by Gram-negative pathogenic bacteria* PLoS One, **10**: e0116896

Porphyromonas gingivalis
Cecil, J.D., O'Brien-Simpson, N.M., Lenzo, J.C., Holden, J.A., Chen, Y-Y., Singleton, W., Gause. K.T., Yan, Y., Caruso, F. and Reynolds, E.C. (2016) *Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens* PLoS One **11**: e0151967

Pseudoalteromonas* -** see ***Shewanella livingstonensis

Pseudomonas aeruginosa
Ballok, A.E., Filkins, L.M., Bomberger, J.M., Stanton, B.A. and O'Toole, G.A. (2014) *Epoxide-mediated differential packaging of Cif and other virulence factors into outer membrane vesicles* J. Bacteriol., **196**, 3633–3642
Bauman, S.J. and Kuehn, M.J. (2006) *Purification of outer membrane vesicles from Pseudomonas aeruginosa and their activation of an IL-8 response* Microbes Infect., **8**, 2400-2408
Bauman, S.J. and Kuehn, M.J. (2009) *Pseudomonas aeruginosa vesicles associate with and are internalized by human lung epithelial cells* BMC Microbiol., **9**:26
Bomberger, J.M., MacEachran, D.P., Coutermarsh, B.A., Ye, S., O'Toole, G.A. and Stanton, B.A. (2009) *Long-distance delivery of bacterial virulence factors by Pseudomonas aeruginosa outer membrane vesicles* PLoS Pathog., **5**:e1000382
Choi, Y., Kwon, Y., Kim, D-K., Jeon, J., Jang, S.C., Wang, T., Ban, M., Kim, M-H., Jeon, S.G. et al (2015) *Gut microbe-derived extracellular vesicles induce insulin resistance, thereby impairing glucose metabolism in skeletal muscle* Sci. Rep., **5**: 15878
Couto, N., Schooling, S.R., Dutcher, J.R. and Barber, J. (2015) *Proteome profiles of outer membrane vesicles and extracellular matrix of Pseudomonas aeruginosa biofilms* J. Proteome Res., **14**, 4207−4222
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Koeppen, K., Hampton, T.H., Jarek, M., Scharfe, M., Gerber, S.A., Mielcarz, D.W., Demers, E.G., Dolben, E.L. et al (2016) *A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles* PloS Pathog., **12**: e1005672
MacDonald, I.A. and Kuehn, M.J. (2013) *Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa* J. Bacteriol., **195**, 2971–2981
MacEachran, D.P., Ye, S., Bomberger, J.M., Hogan, D.A., Swiatecka-Urban, A., Stanton, B.A. and O'Toole, G.A. (2007) *The Pseudomonas aeruginosa secreted protein PA2934 decreases apical membrane expression of the cystic fibrosis transmembrane conductance regulator* Infect. Immun., **75**, 3902-3912
Schooling, S.R., Hubley, A. and Beveridge, T.J. (2009) *Interactions of DNA with biofilm-derived membrane vesicles* J. Bacteriol., **191**, 4097-4102
Tashiro, Y., Sakai, R., Toyofuku, M., Sawada, I., Nakajima-Kambe, T., Uchiyama, H. and Nomura, N. (2009) *Outer membrane machinery and alginate synthesis regulators control membrane vesicle production in Pseudomonas aeruginosa* J. Bacteriol., **191**, 7509-7519
Tashiro, Y., Ichikawa, S., Shimizu, M., Toyofuku, M., Takaya, N., Nakajima-Kambe, T., Uchiyama, H. and Nomura, N. (2010) *Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in Pseudomonas aeruginosa* Appl. Environ. Microbiol., **76**, 3732-3239
Toyofuku, M., Roschitzki, B., Riedel, K. and Eberl, L. (2012) *Identification of proteins associated with the Pseudomonas aeruginosa biofilm extracellular matrix* J. Proteome Res., **11**, 4906−4915
Toyofuku, M., Zhou, S., Sawada, I., Takaya, N., Uchiyama, H. and Nomura, N. (2014) *Membrane vesicle formation is associated with pyocin production under denitrifying conditions in Pseudomonas aeruginosa* PAO1 Environ. Microbiol., **16**, 2927–2938
Turnbull, L., Toyofuku, M., Hynen, A.L., Kurosawa, M., Pessi, G., Petty, N.K., Osvath, S.R., Cárcamo-Oyarce, G. et al (2016) *Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms* Nat. Comm., **7**: 11220
Zhao, K., Deng, X., He, C., Yue, B. and Wu, M. (2013) *Pseudomonas aeruginosa outer membrane vesicles modulate host immune responses by targeting the Toll-like receptor 4 signaling pathway* Infect. Immun., **81**, 4509-4518

Psychrobacter fozii* - *see* *Shewanella livingstonensis

Salmonella enterica

Bai, J., Kim, S.I., Ryu, S. and Yoon, H. (2014) *Identification and characterization of outer membrane vesicle-associated proteins in Salmonella enterica Serovar Typhimurium* Infect. Immun., **82**, 4001–4010
Bonnington, K.E. and Kuehn, M.J. (2016) *Outer membrane vesicle production facilitates LPS remodeling and outer membrane maintenance in Salmonella during environmental transitions* mBio, **7**: e01532-16
Daleke-Schermerhorn, M.H., Felix, T., Soprova, Z., ten Hagen-Jongman, C.M., Vikström, D., Majlessi, L., Beskers, J., Follmann, F., de Punder, K., van der Wel, N.N. et al (2014) *Decoration of outer membrane vesicles with multiple antigens by using an autotransporter approach* Appl, Environ. Microbiol., **80**, 5854–5865
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Liu, Q., Liu, Q., Yi, J., Liang, K., Liu, T., Roland, K.L., Jiang, Y. and Kong, Q. (2016) *Outer membrane vesicles derived from Salmonella Typhimurium mutants with truncated LPS induce cross-protective immune responses against infection of Salmonella enterica serovars in the mouse model* Int. J. Med. Microbiol., **306**, 697–706
Muralinath, M., Kuehn, M.J., Roland, K.L. and Curtiss III, R. (2011) *Immunization with Salmonella enterica serovar Typhimurium-derived outer membrane vesicles delivering the pneumococcal protein PspA confers protection against challenge with Streptococcus pneumoniae* Infect. Immun., **79**, 887–894

Salmonella typhimurium

Liu, Q., Liu, Q., Yi, J., Liang, K., Liu, T., Roland, K.L., Jiang, Y. and Kong, Q. (2016) *Outer membrane vesicles derived from Salmonella Typhimurium mutants with truncated LPS induce cross-protective immune responses against infection of Salmonella enterica serovars in the mouse model* Int. J. Med. Microbiol., **306**, 697–706

Shewanella livingstonensis

Frias, A., Manresa, A., de Oliveira, E., López-Iglesias, C. and Mercade, E. (2010) *Membrane vesicles: a common feature in the extracellular matter of cold-adapted Antarctic bacteria* Microb. Ecol., **59**, 476–486

Shewanella veisiculosa

Pérez-Cruz, C., Carrión, O., Delgado, L., Martinez, G., López-Iglesias, C. and Mercade, E. (2013) *New type of outer membrane vesicle produced by the Gram-negative bacterium Shewanella vesiculosa M7T: implications for DNA* Appl. Environ. Microbiol., **79**, 1874-1881

Tannerella forsythia

Cecil, J.D., O'Brien-Simpson, N.M., Lenzo, J.C., Holden, J.A., Chen, Y-Y., Singleton, W., Gause. K.T., Yan, Y., Caruso, F. and Reynolds, E.C. (2016) *Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens* PLoS One **11**: e0151967

Veith, P.D., Chen, Y-Y., Chen, D., O'Brien-Simpson, N.M., Cecil, J.D., Holden, J.A., Lenzo, J.C. and Reynolds, E.C. (2015) *Tannerella forsythia outer membrane vesicles are enriched with substrates of the type IX secretion system and TonB-dependent receptors* J. Proteome Res., **14**, 5355−5366

Treponema denticola

Cecil, J.D., O'Brien-Simpson, N.M., Lenzo, J.C., Holden, J.A., Chen, Y-Y., Singleton, W., Gause. K.T., Yan, Y., Caruso, F. and Reynolds, E.C. (2016) *Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens* PLoS One **11**: e0151967

Vibrio cholerae

Elluri, S., Enow, C., Vdovikova, S., Rompikuntal, P.K., Dongre, M., Carlsson, S., Pal, A., Uhlin, B.E., Wai, S.N. (2014) *Outer membrane vesicles mediate transport of biologically active Vibrio cholerae cytolysin (VCC) from V. cholerae strains* PLoS One, **9**: e106731

Mondal, A., Tapader, R., Chatterjee, N.S., Ghosh, A., Sinha, R., Koley, H., Saha, D.R., Chakrabarti, M.K., Wai, S.N. and Pala, A. (2016) *Cytotoxic and inflammatory responses induced by outer membrane vesicle-associated biologically active proteases from Vibrio cholerae* Infect. Immun., **84**, 1478-1490

Roier, S., Zingl, F.G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T.O., Kiug, L., Gadermaier, B. et al (2016) *A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria* Nat. Comm., **7**: 10515

Sjöström, A.E., Sandblad, L., Uhlin, B.E. and Wai, S.N. (2015) *Membrane vesicle-mediated release of bacterial RNA* Sci. Rep., **5**: 15329

Vibrio tasmaniensis

Vanhove, A.S., Duperthuy, M., Charrière, G.M., Le Roux, F., Goudenège, D., Gourbal, B., Kieffer-Jaquinod, S., Couté, Y., Wai, S.N. and Destoumieux-Garzón, D. (2015) *Outer membrane vesicles are vehicles for the delivery of Vibrio tasmaniensis virulence factors to oyster immune cells* Environ. Microbiol., **17**, 1152–1165

Yersinia pestis

Eddy, J.L., Gielda, L.M., Caulfield, A.J., Rangel, S.M. and Lathem, W.W. (2014) *Production of outer membrane vesicles by the plague pathogen Yersinia pestis* PloS One, **9**: e107002

3b. Gram-positive bacteria and mycobacteria

Bacillus anthracis

Wolf, J.M., Rivera, J. and Casadevall, A. (2012) *Serum albumin disrupts Cryptococcus neoformans and Bacillus anthracis extracellular vesicles* Cellular Microbiology (2012) 14(5), 762–773

Bacillus subtilis

Brown, L., Kessler, A., Cabezas-Sanchez, P., Luque-Garcia, J.L. and Casadevall, A. (2014) *Extracellular vesicles produced by the Gram-positive bacterium Bacillus subtilis are disrupted by the lipopeptide surfactin* Mol. Microbiol., **93**, 183–198

Lee, E-Y., Choi, D-Y., Kim, D-K., Kim, J-W., Park, J O., Kim, S., Kim, S-H., Desiderio, D.M., Kim, Y-K., Kim, K-P- and Gho, Y.S. (2009) *Gram-positive bacteria produce membrane vesicles: Proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles* Proteomics, **9**, 5425-5436

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Clostridium perfringens

Jiang. Y., Kong, Q., Roland, K.L. and Curtiss III, R. (2014) *Membrane vesicles of Clostridium perfringens type A strains induceinnate and adaptive immunity* Int. J, Med. Microbiol., **304**, 431–443

Mycobacterium tuberculosis

D'Lima, N.G. and Teschke, C.M. (2015) *A method to investigate protein association with intact sealed mycobacterial membrane vesicles* Anal. Biochem. **485**, 109–111

Lee, J., Kim, S-H., Choi, D-S., Lee, J.S., Kim, D-K., Go, G., Park, S-M., Kim, S.H., Shin, J.H., Chang, C.L. and Gho, Y.S. (2015) *Proteomic analysis of extracellular vesicles derived from Mycobacterium tuberculosis* Proteomics, **15**, 3331–3337

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Mini-Review MS16; 3rd edition, March 2017