THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITÉ GRENOBLE ALPES

École doctorale : CSV- Chimie et Sciences du Vivant
Spécialité : Virologie - Microbiologie - Immunologie
Unité de recherche : Institut de Biologie Structurale

Cartographie du proxisome d'ExoU, un effecteur du système de sécrétion de type III de Pseudomonas aeruginosa

Mapping the Host Proxisome of ExoU, a Type III Secretion System effector of Pseudomonas aeruginosa

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Acknowledgments

I would like to thank first the jury members, for agreeing with enthusiasm to be part of this milestone and to evaluate my work. My reporters Undine Mechold and Etienne Meunier, the examinateur Eric Cascales. A special thanks to Corinne Mercier, the president of the jury who has accompanied me throughout the past five years and watched me evolve as a scientist. You have supported me on my bad days and I have learned so much from you. She was also part of my CSI committee along with Laurence Aubry and Cécile Morlot, who have encouraged me and listened to me during the yearly meetings, got invested in project and helped shape what I am presenting here. Thank you for making this journey smoother than what it could have been.

Now it is time for my favorite duo, my supervisors Ina and Eric. Eric, you are very smart it is impressive, the way you think and come up with ideas, your involvement in many scientific projects covering various aspects in addition to the non-scientific ones that are for the benefit of the community and the environment. You inspired me to work for the community too, pushed me to speak up and taught me a lot scientifically and personally. Ina, you are smart too! you have achieved so much in your career, and you continue to reach higher levels. You have been good to me, both scientifically and personally, we had many good moments that I'll remember for a long time and we laughed a lot. You were there whenever I needed you and I have learned so much from you in different contexts. You are a woman of power and that, I aspire to be. You guys gave me a balanced amount of independence and supervision. You both are good and caring human beings, which is as or even more important than being good scientifically, this played a huge part in me being happy and comfortable during this PhD. You supported me, I was able to communicate my feelings which you embraced and accepted as they are all while giving me your honest opinion. You listened and tolerated me in all my states: angry, happy, frustrated, worried, stressed, excited, energetic or when I am joking and being goofy. Lastly, you remained positive all the way. I am lucky to have had you both by my side during this once in a lifetime experience and made my writing period smooth with your input and corrections. Despite and because of everything we've been through, I wouldn't have wanted it any other way. En gros, if I can just pick up the best of both your brains, I would make a top scientist in the field in addition to becoming a better person!

To my PBRC family, thank you for everything and for all the memories we made, I will hold on to them as long as I can. You have shown me constant respect, love and care. Stéphanie, you came back at the right time and helped me on and off the bench, which is exactly what I needed. Thank you for your efforts, your presence and for all the diverse conversations we had and the moments we shared. François, you are the reason I came to PBRC in 2022 as

part of the GRAL scholarship, little did I know it will turn out like that! After my M2, you kept on looking out for me and asking about my project and I appreciate that. I will miss the nature briefing discussions every other morning during coffee. Sylvie and Mylène, you took care of me, showed me love and supported me scientifically and personally in every possible situation. You taught me a lot at the lab in addition to delicious recipes and introduced me to many board games! Marie-Claire, thank you for what you have taught me and for listening to me. Viviana and Antoine, thank you for caring and for the interesting discussions we had. I thank mes jeunes préférés, Fabien, Hortense, Loréline and Eva for their humor, company and for all the talking and listening we did. I can't forget the PBRC PhD alumni, Manon, Aimé and Victor who were there since I arrived and made my lab life better. Manon, you were and are still supporting me despite the distance. Lastly, Ina was happy when she was recruiting Lama and said "we might be recruiting a Lebanese girl, I hope you become friends". Well, I am glad this happened. It was a pleasure to share the lab and the office with you, we made so many memories and laughed a lot, we ate healthy, we went to gym, we gossiped, cared for and always supported each other and I knew you were always there. I will hold on to that, thank you for everything.

I would also like to thank Jean-Philippe Kleman from the M4D platform who has accompagnied me since my M2 and was patient with me while teaching me flow cytometry and fluorescence microscopy. Christine Chatellard from the EBEV group, thank you for getting involved in the project, for your passion, valuable insights and for teaching me everything I know about vesicles!

To my second floor colleagues, thank you for the sweet and genuine daily interactions, all the outings we planned and the fun we had. Special thanks to Massilia, Perrine and María from the office 369 for their precious presence. Perrine, you have been an amazing office mate and friend in the past four years, you are a great listener and supporter and a good-hearted person, thank you for all the moments we shared. María, you unexpectedly changed my experience at the lab as well as in Grenoble, we had so much fun and did a lot of things, stood by each other when needed and reminded each other of our worth. May we make many more memories, thank you!

To my PhD, post-doc and lab fellows, Francesca and Sara, my two Italian friends at the IBS, thank you for all the memories and for your support. Borys, you are a great frined and I always enjoy the time we spend together. Yara, Rida and Rim, thank you for being good to me and checking up on me whenever we cross. Juneina, thank you for being very supportive especially in the past few crazy months, I appreciate you and our friendship.

Elliott, you have been an amazing flatmate and showed me care and support in the past few months. This is precious and I will always remember it. You made it easy to live with someone I didn't know before, thank you!

Rebecca and Laetitia. Rebecca, it took us time to become friends, but we made it! I am glad we became in each other's lives. You and Laetitia have been quite supportive and present throughout the past two years with everything that has been happening in the world. Thank you for all the fun and memories we shared. Looking forward!

My Lebanese friends wherever you are, Nour, Ithamar, Maria, Carla, Nouryaz, Marion, Marwa, Rim, Mariam, the two Ayas, Joseph, Assile and Tatiana in addition to my high school besties Hiba and Linda. Thank you for being by my side all this time, for all the moments and laughters we shared, for every time you checked on me. Each of you has supported me in their own special way and that is precious.

Zwaiz, thank you for your support and being part of my journey.

My Grenoble peeps. Hadi, you have been here since my first day in Grenoble, you have been an amazing friend. I love when we just spend time doing nothing and more recently playing fifa. Rouba, Pier, Eliane, Roy and Elias, thank you for everything, every time I see you I feel better even if we do nothing and this is priceless.

My master 2 gems, Silvia and Martina. So lucky to have been able to share the journey with you since 2020. We experienced everything together, we shared so many memories, we celebrated each other on every occasion. This is such a blessing! Martina, you are the last one and we are waiting for you.

I also thank my family, my grandmother, my uncles, my aunts and my cousins: Elise, Edwige, Christy-Joy, Justine, Thea-Maria and Romy for their endless support.

When I was 17, I decided that I am going to study biology and then I said if I pursue it, I want to do a master and a PhD. And it worked out, now eight years later I got my PhD. I was inspired during my school years by my teacher, Dolly, who inspired me through six years with her love and passion for biology and if it wasn't for her, maybe I wouldn't have loved the subject, so I thank you for this Dolly. Also, to share this with you, everyone, after everything that happened throughout these four years, it is priceless to have you all here, to see you all to be with you and to share this special moment.

Rhea, we got to live together during my stay in France for two years. You were my best friend and now you are my sister. Living together changed the dynamics and the relationship, brought

us closer than ever and for that I'm grateful. I'm so happy I got to live with my best friend as we may never be able to do that again.

Rasha and Chris, you took me in since the first day I arrived in Grenoble, you made me feel good, you made me feel better, you supported me every day every time anytime. You treated me as family since the first day I laid feet in Grenoble, until you became my family and I can never thank you enough. We celebrated so much in these five years and may we continue to do so for many more years to come.

Rey, my brother, despite our differences, you always believed in me, supported me, played an important part in me coming here to pursue my masters and pushed for it, and always want me to have the best of the best of what is possible. I wish you could have been here and I miss you.

Mom and dad, you gave me your all, everything you had, sometimes even more, with no limits. Listened to me every time I wanted to talk, even when I called at 3-4-5 am during my M2. I owe you everything that I am and I have achieved, the woman that I am is because of you.

Thank you to each and everyone for being part of my journey in their own way. It is with your support and love that I was able to push myself to exceed my limits and reach where I am today.

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Abbreviations

aa	Amino acid
ADPRT	ADP ribosyl transferase
ANCL	Adult neuronal ceroid lipofuscinosis
AIDS	Acquired immunodeficiency syndrome
AT	Autotransporters
ATG	Autophagy-related genes
AMR	Antimicrobial resistance
ANI	Average nucleotide identity
APEX	Ascorbate peroxidase
α-syn	α-synuclein
BL	Biotin ligase
BSIs	Blood stream infections
β-lactams	Beta-lactams
BioAMP	Biotinoyl-5'-AMP
BioID	Biotin identification
c-AMP	Cyclic-AMP
CBD	Chaperone binding domain
c-di-GMP	Cyclic-di-GMP
CF	Cystic fibrosis
CMA	Chaperone-mediated autophagy
CFTR	CF transmembrane conductance regulator
c-ring	Cytoplasmic ring
CCV	*Coxiella*-containing vacuoles
CGN	Cis-Golgi network
CbpE	Chitin-binding-protein E
CNF1	Cytotoxic necrotizing factor 1
COPD	Chronic obstructive pulmonary disorder
DAG	Diacylglycerol

EE	Early endosome
EEA1	Early endosome antigen 1
E. coli	*Escherichia coli*
ER	Endoplasmic reticulum
ERES	ER exit sites
ERGIC	ER-Golgi intermediate compartment
ESCRT	Endosomal sorting complexes required for transport
EGTA	Ethylene Glycol Tetraacetic Acid
eDNA	Extracellular DNA
EPS	Exopolysaccharides
ETA	Exotoxin A
EF-2	Elongation factor 2
EVs	Extracellular vesicles
Est	Esterase
ExlBA	Exolysin BA
f-actin	Filamentous actin
GAP	GTPase-activating protein
GTP	Guanosine triphosphate
HAP	Hospital acquired pneumonia
HK	Histidine kinase
Hcp	Hemolysin coregulated protein
iFAST	Improved fluorescence activating and absorption shifting tag
IFN-γ	Interferon gamma
ILVs	Intraluminal vesicles
IL	Interleukin
IP3	Inositol (1,4,5)-trisphosphate
IM	Inner membrane
KD	Knock-down
KO	Knock-out

LE	Late endosome
LBPA	Lysobisphosphatidic acid
LC	Liquid chromatography
LPS	Lipopolysaccharides
LAMP1	Lysosome associated membrane protein-1
Las	Elastase
Lip	Lipase
MAC	Membrane attack complex
MAPS	Misfolding associated protein secretion
Mex	Multidrug efflux pumps
MLD	Membrane localization domain
MS	Mass spectrometry
MVBs	Multivesicular bodies
OM	Outer membrane
OmpA	Outer membrane protein A
OMVs	Outer membrane vesicles
Pa	*Pseudomonas aeruginosa*
PE	Phosphatidylethanolamine
PAPI-2	*P. aeruginosa* pathogenicity island 2
PI	Propidium iodide
PI(4,5)P2	Phosphatidylinositol-4,5-bisphosphate
PLA2	Phospholipase A2
PM	Plasma membrane
PL	Proximity labeling
PLA	Proximity labeling assay
PLA2	Phospholipase A2
POI	Protein of interest
PLC	Phospholipase C
PLP	Patatin-like phosphatase

PI3P	Phosphatidylinositol 3-phosphate
PI4P	Phosphatidylinositol-4-phosphate
PRR	Pattern recognition receptors
PAMPs	Pathogen associated molecular patterns
PARs	Protease-activated receptors
RND	Resistance-nodule-dissection
RE	Recycling endosome
RalGDS	Guanine exchange factor for Ral GTPases
SCV	*Salmonella*-containing vacuole
SPI	*Salmonella* pathogenicity island
SNPs	Single nucleotide polymorphisms
SNARE	Soluble N-ethylmaleimide sensitive factor receptor
SIFs	*Salmonella*-induced filaments
STX	Syntaxin
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor alpha
T4P	Type IV pili
T1SS	Type 1 Secretion System
T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4BSS	Type 4B Secretion System
T5SS	Type 5 Secretion System
T6SS	Type 6 Secretion System
TGN	Trans-Golgi network
TPS	Two partner secretion
TCS	Two-component system
UTI	Urinary tract infection
vATPase	v-type vacuolar H^+ ATPase
Vsp34	Vacuolar protein sorting 34

VAMP	Vesicle-associated membrane protein
VAP	Ventilator-associated pneumonia
VE-cadherin	Vascular endothelium cadherin
Vfr	Virulence factor regulator
Vice	Vacuole-inducing *Coxiella* effector
WHO	World health organization
WT	Wild-type

Introduction

Chapter 1 - Meet *Pseudomonas aeruginosa*

1.1. *Pseudomonas aeruginosa*: diversity and adaptability

1.1.1 The *P. aeruginosa* species

P. aeruginosa is a Gram-negative rod-shaped opportunistic pathogen and is around 1-5 µm long and 0.5-1 µm wide (Diggle & Whiteley, 2020). *P. aeruginosa* was first isolated by C. Gessard in 1882 who was a French military pharmacist. Gessard identified *P. aeruginosa* after noticing the blue color, caused by the pyocyanin pigment, in skin wounds (Gessard, 1882). Since then, *P. aeruginosa* has been the center of many studies concerning its virulence factors, antibiotic resistance, phylogenetic diversity and the different types of infections it can cause. The first sequenced *P. aeruginosa* genome was the one of the PAO1 laboratory strain of 6.3 Mbp coding for 5572 genes (Stover et al., 2000). Today, more than 8000 *P. aeruginosa* strains were sequenced providing a huge database concerning its genetic diversity (www.pseudomonas.com) and making it one of the most studied bacterial species (Mathee et al., 2008; Winsor et al., 2016). This bacterium could be found in different environments including soil, water, plants, animals and humans (**Figure 1**, from (Scales et al., 2014) as it is able to survive in temperatures ranging between 4 to 42°C (Diggle & Whiteley, 2020) and a wide interval of pH (4.5-9.0) (Khan et al., 2010). Genomes of *P. aeruginosa* strains range between 5.5 and 7.5 Mbp (Ozer et al., 2019) and they include more than 500 regulatory genes (Stover et al., 2000; Galán-Vásquez et al., 2011). This characteristic allows *P. aeruginosa* to survive and adapt differently to the diverse encountered environments.

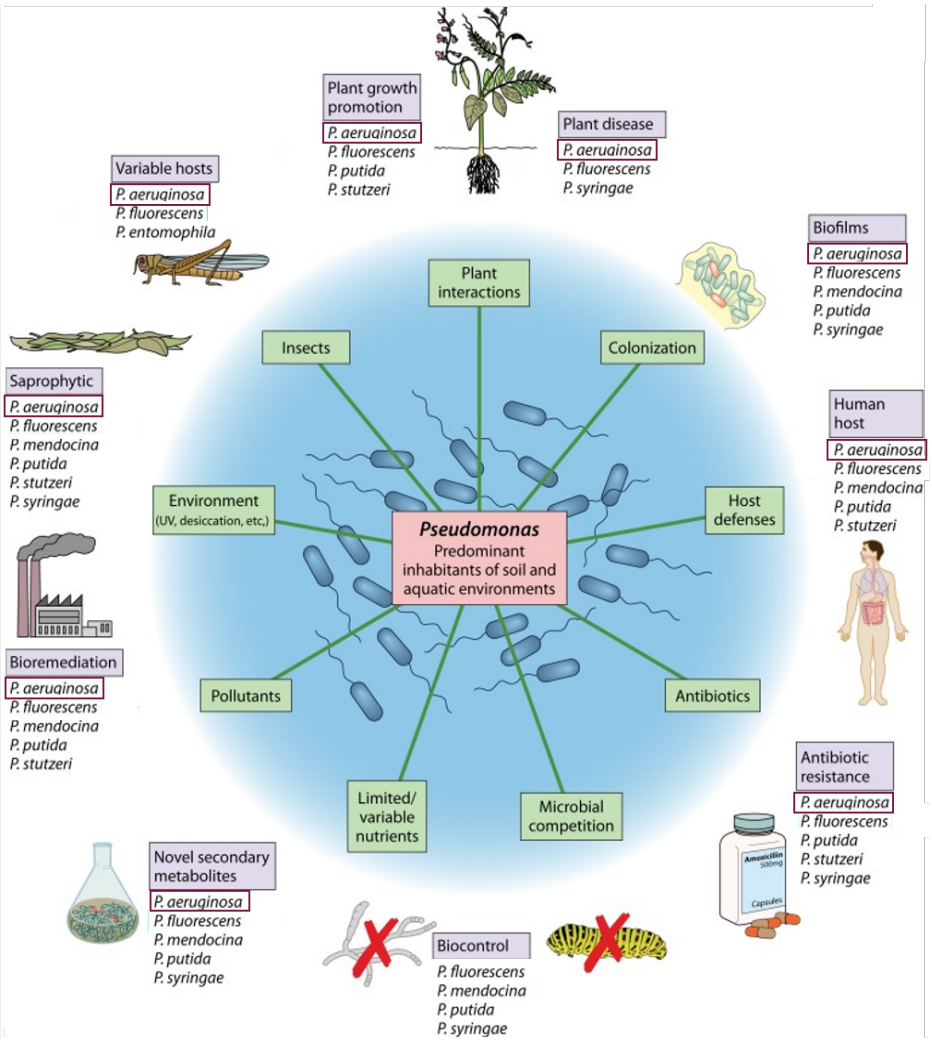

Figure 1. Environmental niches of the *Pseudomonas* genus with a focus on the wide distribution of *P. aeruginosa*. From (Scales et al., 2014).

1.1.2 *P. aeruginosa* phylogenetic diversity

Throughout the years, *P. aeruginosa* isolates have been classified first into three groups, 1 to 3, each with its respective model strain PAO1, PA14 and PA7 (Stewart et al., 2014). The classification was then better refined, due to the increase in the number of sequenced genomes, into 5 phylogenetic groups based on their single nucleotide polymorphisms (SNPs) and average nucleotide identity (ANI) (Freschi et al., 2019). Groups 1 and 2 include 98% of total isolates (n= 986 and 297 respectively). Group 3 (n=14) is clearly distant from groups 1 and 2. Lastly, groups 4 and 5, previously associated with group 3, are in an intermediate position between groups 1-2 and group 3 (**Figure 2**). A key distinction between these phylogroups is in terms of virulence factors specifically the Type 3 Secretion System (T3SS) (Freschi et al., 2019; Ozer et al., 2019). The T3SS is a needle-like structure that injects 4 toxins into host cell cytoplasm (the T3SS and its toxins are detailed in chapter 2). Groups 1, 2 and 4 are T3SS$^+$ but harbor different toxins : ExoS, ExoT and ExoY for group 1, ExoU instead of

ExoS for group 2 and only ExoS and ExoT for group 4 (Freschi et al., 2019; Ozer et al., 2019). On the other hand, groups 3 and 5 are T3SS$^-$ but have peculiar virulence factors genes. Indeed, the two partner secretion (TPS) system Exolysin A and B (ExlBA) were identified in these strains. ExlB transports ExlA through the bacterial outer membrane (OM) and, once secreted, ExlA's pore-forming activity is triggered leading to host cell death (Elsen et al., 2014; Reboud et al., 2017). In addition, a third type 2 secretion system was identified in group 3 and termed Txc for third Xcp homolog (Cadoret et al., 2014). Due to the divergence of the genome sequences of the strains compared to the ones of group 1 and 2, group 3 strains are considered as a taxonomic outlier and were was recently reclassified as *P. paraeruginosa* (Rudra et al., 2022). Due to the large repertoire of available information on *P. aeruginosa*, I will focus in this chapter on the infections caused by *P. aeruginosa*, its lifestyles, secretion systems and major virulence factors.

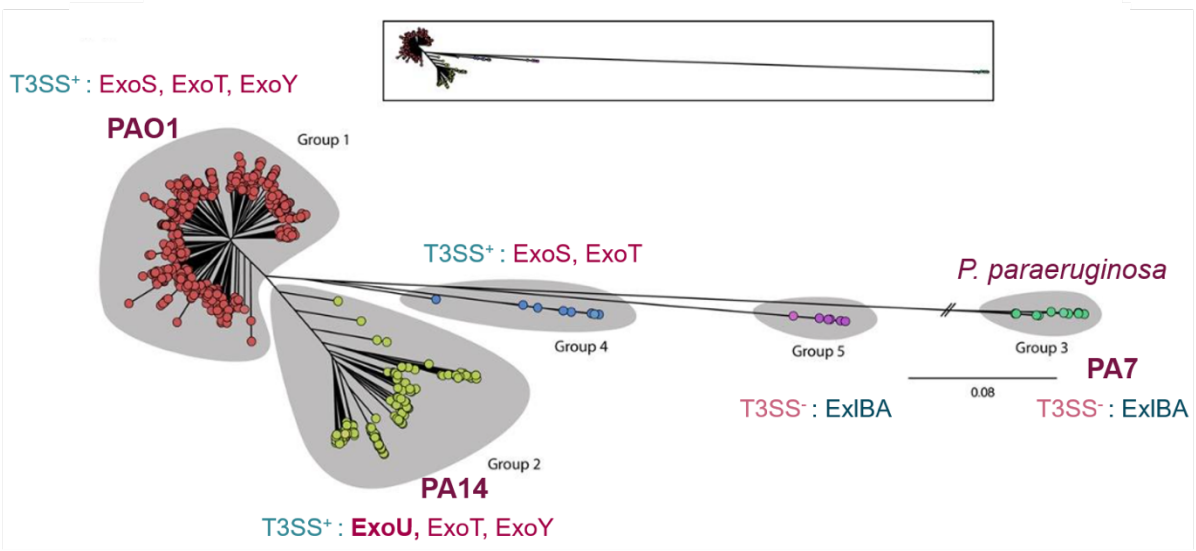

Figure 2. Phylogenetic tree of *P. aeruginosa*.
The five groups of the isolates are highlighted by gray areas (n=1311). Each group is identified by a specific color: group 1 (red), group 2 (green), group 3 (aquamarine), group 4 (blue) and group 5 (violet). Groups 1, 2 and 3 are presented by the strains PAO1, PA14 and PA7 respectively. The absence or presence of the T3SS and its toxins is indicated. Group 3 is considered as *P. paraeruginosa*. The tree on the top shows the real genetic distance between the isolates of group 3 and the other groups. Adapted from (Freschi et al., 2019).

1.1.3 *P. aeruginosa* infections and lifestyles

1.1.3.1. Planktonic *vs* biofilm states and associated infections

P. aeruginosa is a leading cause of nosocomial infections worldwide. This bacterium takes advantage of immunosuppressed patients including cancer and acquired immunodeficiency syndrome (AIDS) patients (S. J. Wood et al., 2023) with a mortality rate up to 40%. *P. aeruginosa* is able to cause a variety of short-term acute and long-term chronic infections that are linked to the lifestyle adopted by the pathogen upon infection (Furukawa et al., 2006).

P. aeruginosa has two distinct well-defined lifestyles: motile (planktonic) and sessile (biofilm). During the planktonic state, *P. aeruginosa* is a free-living bacterium, motile and expressing certain virulence factors (David et al., 2024). For instance, in the case of acute lung infection, it attaches to the mucosal barrier using the flagella and the type IV pili (T4P) which are two adhesins that stimulate the inflammatory response of the host (Gellatly & Hancock, 2013). Once adhered, *P. aeruginosa* secretes T3SS effectors to avoid host phagocytosis and then to damage and breach the epithelial barrier. In addition, it secretes through the Type 2 Secretion System (T2SS) a protease, LasB, which cleaves both the extracellular matrix components and the vascular endothelium cadherin (VE-cadherin) disrupting the junctions between neighboring cells (Golovkine et al., 2014; Huber et al., 2014, 2014). Evidently, the cumulative effects of these effectors allow *P. aeruginosa* to grow, reach the bloodstream and then disseminate throughout the whole body of the host reaching other organs (Golovkine et al., 2014). Therefore, this lifestyle manifests with the most severe symptoms and lesions associated with poor outcomes. *P. aeruginosa* acute infections include ventilator-associated pneumonia (VAP) and hospital acquired pneumonia (HAP), eye infections, skin infections (burn wound), blood stream infections (BSIs), urinary tract infection (UTI), endocarditis and osteomyelitis (Armour et al., 2007; Green et al., 2008; S. J. Wood et al., 2023). Indeed, if not treated well and on time, acute infections can become chronic (**Figure 3**).

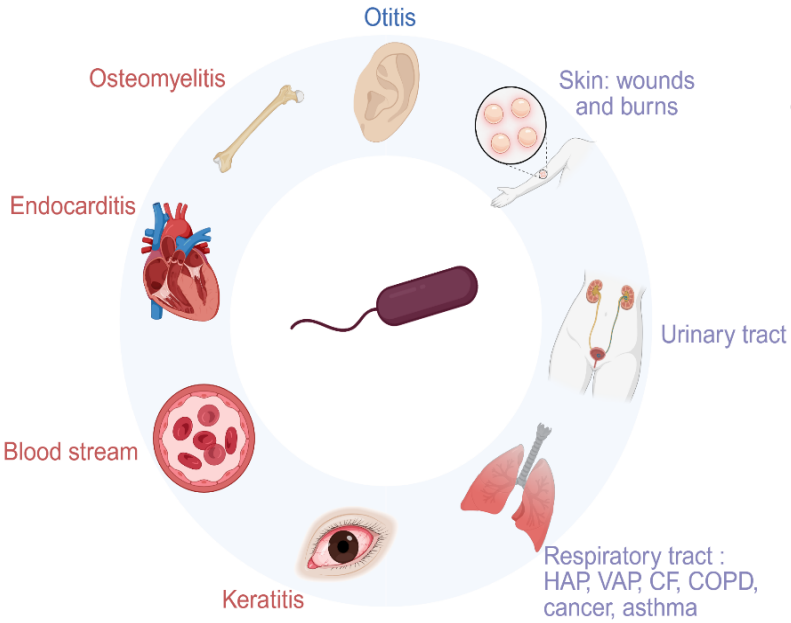

Figure 3. Diversity of infections caused by *P. aeruginosa*.

The different infections caused by *P. aeruginosa*. Acute infections are shown in red, chronic in blue and those that can be both are in purple. HAP: hospital acquired pneumonia, VAP: ventilator-associated pneumonia, CF: cystic fibrosis and COPD: chronic obstructive pulmonary disorder. Made with BioRender (www.biorender.com)

Conversely, when in a sessile state, the bacteria gradually forms biofilm. This begins with the bacteria's attachment (reversible) to a biotic or abiotic surface mediated by the flagellum followed by a stronger adsorption (irreversible) to the surface facilitated by the T4P (David et al., 2024). *P. aeruginosa* progressively produces the biofilm matrix composed of

exopolysaccharides (EPSs), extracellular DNA (eDNA), proteins, lipids and outer membrane vesicles (OMVs) leading to the formation of microcolonies (Y. Li et al., 2020). These structures grow in biomass to from 3D macrocolonies and eventually a mature biofilm embedded within the matrix. Within the biofilm community, the matrix protects the bacteria allowing it to persist in the chronically infected host (David et al., 2024; Y. Li et al., 2020; Valentini et al., 2018). Bacteria in biofilms have a repressed T3SS and motility appendages while the T6SS is upregulated (Baraquet & Harwood, 2013; Moscoso et al., 2011; M. Zhu et al., 2016). The biofilm can remain in that state or initiate a dispersion during which a rearrangement in the structure takes place, releasing bacteria that could disseminate in a new environment or repeat the cycle at the same site (**Figure 4**) (Guilhen et al., 2017). *P. aeruginosa* chronic infections include lung infection of patients with cystic fibrosis (CF), chronic obstructive pulmonary disorder (COPD), chronic asthma and lung cancer (Holm et al., 2013; S. J. Wood et al., 2023; Q. Zhang et al., 2012). CF is a recessive genetic disorder manifesting by a defective anion channel, the CF transmembrane conductance regulator (CFTR). In both, CF and COPD, thick mucus is overproduced leading to a favorable environment for *P. aeruginosa* colonization and biofilm formation (Flume & Van Devanter, 2012). *P. aeruginosa* causes chronic ear (otitis media) and wound infections in addition to UTIs as it is able to form biofilm on urinary catheters (**Figure 3**) (Mansoor et al., 2009; Mittal et al., 2009; Serra et al., 2015).

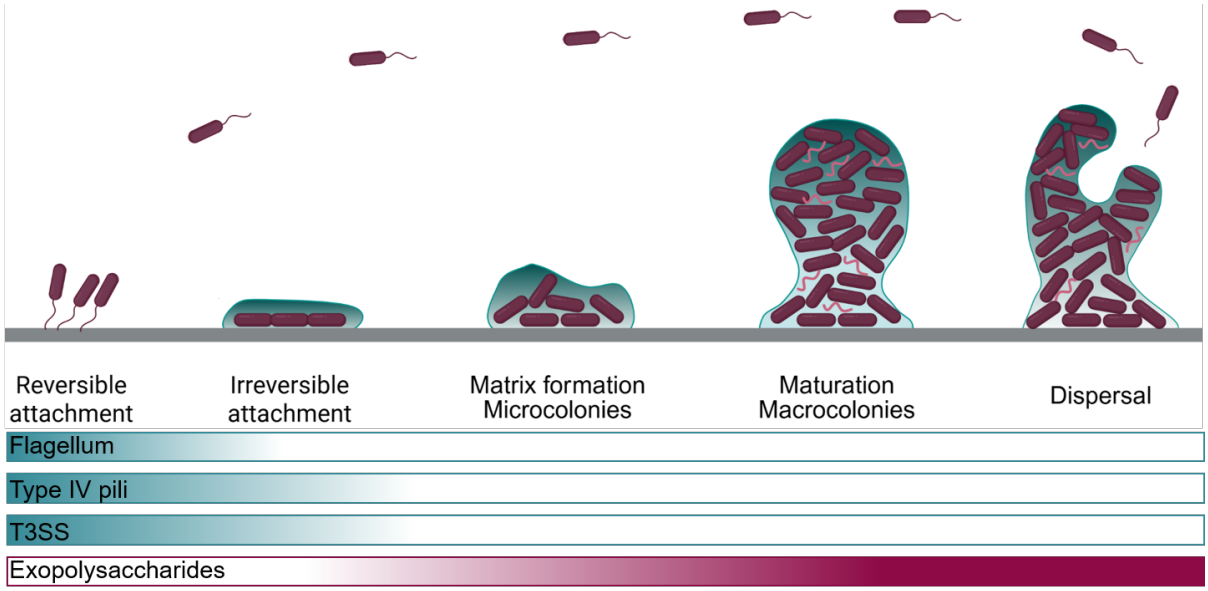

Figure 4. The biofilm formation cycle of *P. aeruginosa*.
The different steps of biofilm formation in the life cycle of *P. aeruginosa* are depicted from attachment to maturation and then dispersal. Pink: eDNA and green: exopolyschaccharides. The formation of biofilm is accompanied with the decrease of expression of the flagellum, Type IV pili and T3SS while exopolysaccharides production increases. Made with biorender.

1.1.3.2. Regulatory switch between the two lifestyles

The switch between the two lifestyles (sessile *vs* biofilm) is modulated by the Gac/Rsm cascade. This regulatory network requires the two-component system (TCS) GacS/GacA

(Francis et al., 2017). TCSs are made of a histidine kinase (HK) that activates its cognate response regulator (such as transcription factors) upon specific signal sensing (Gao & Stock, 2009). The GacS/GacA TCS is modulated by two HKs, LadS and RetS. RetS represses the system while LadS activates it upon high calcium sensing (Broder et al., 2016; Francis et al., 2017). When inactive, RetS inhibits GacS allowing the RsmA RNA-binding protein to inhibit biofilm formation leading to the subsequent expression of genes responsible for the planktonic lifestyle. In the ON state, GacS phosphorylates GacA leading to the induction of the transcription of two small non-coding RNAs, RsmZ and RsmY, which bind and inhibit RsmA allowing the expression of biofilm formation genes (Bordi et al., 2010; Jennings et al., 2015; Lapouge et al., 2008) and reviewed by (Valentini et al., 2018). This system plays a major and critical role in controlling bacterial cell adhesion during biofilm formation (Wolska et al., 2016).

In parallel, two secondary messengers are modulated: cyclic-di-GMP (c-di-GMP) and cyclic-AMP (c-AMP). The c-di-GMP is synthesized from two guanosine triphosphate (GTP) molecules and its regulation requires a complex network of around 40 proteins (Moscoso et al., 2014; Valentini & Filloux, 2016). *P. aeruginosa* biofilms contain high levels of c-di-GMP compared to the planktonic cells (75-110 *vs* 30 pmol/mg respectively) (Basu Roy & Sauer, 2014). The increased c-di-GMP is used by *P. aeruginosa* to positively induce the production of EPSs and other biofilm determinants (Römling et al., 2013). Whereas, cAMP induces *P. aeruginosa* virulence factors, by binding and activating the virulence factor regulator (Vfr), during the planktonic state (**Figure 5**) (Wolfgang et al., 2003). In addition, the Gac/Rsm cascade and c-di-GMP signaling are connected, as RsmA represses SadC which produces c-di-GMP (Moscoso et al., 2014). A recent study highlighted, on a single cell level, the virulence factors crosstalk in *P. aeruginosa*. It focuses on the dynamics between the c-di-GMP and the T3SS, flagellum and the T6SS all at once. Cells have heterogeneous c-di-GMP levels that directly affect the different secretion systems within subpopulations. They also revealed an antagonistic relationship between the T3SS and the T6SS at the single cell level (H. Chen et al., 2025).

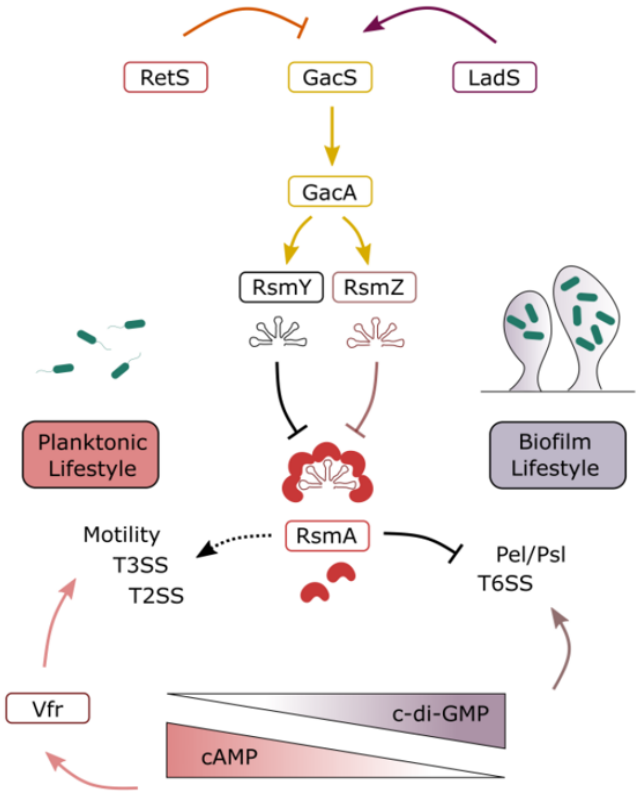

Figure 5. The regulatory switch network controlling *P. aeruginosa's* lifestyle.
The molecular mechanisms governing the switch between the biofilm and sessile lifestyles of *P. aeruginosa*. The cascade of events includes the Gac/Rsm two-component system, c-di-GMP and cAMP levels and modulation of virulence factors expression. From the thesis of Manon-Janet Maitre, 2022

1.2. *P. aeruginosa* antibiotic resistance

Antimicrobial resistance (AMR) is a current major health concern as in 2021, it was estimated that 4.71 million deaths were associated with AMR (Naghavi et al., 2024). Among the causative pathogens, *P. aeruginosa* was responsible for an average of 10% of the AMR-attributable deaths between the years 1990-2021 (Naghavi et al., 2024). The most alarming species concerning the AMR are the ESKAPE pathogens : *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa and enterococcus spp* (Ikuta et al., 2022). *P. aeruginosa* has been a threatening multidrug-resistant pathogen and the carbapenem resistant strains were classified in 2024, by the world health organization (WHO), as a high priority pathogen for which novel antibiotics are urgently required (*WHO Bacterial Priority Pathogens List 2024*, 2024). The mechanisms governing the AMR of *P. aeruginosa* could be divided into three categories: 1) intrinsic resistance 2) adaptive resistance and 3) acquired resistance.

1.2.1. Intrinsic resistance

P. aeruginosa is naturally resistant to several antibiotics owing it to the possession of efflux pumps and to its decreased outer membrane (OM) permeability (**Figure 6**) (Breidenstein et al., 2011). Efflux pumps play a major role in *P. aeruginosa* AMR as they can eliminate molecules belonging to different classes of antibiotics such as beta-lactams (β-lactams),

fluoroquinolones and tetracyclines (Du et al., 2018). One of the most studied family of pumps is the resistance-nodule-dissection (RND) which includes genes encoding for multidrug efflux pumps (Mex) (Dreier & Ruggerone, 2015). For instance, the MexAB-OprM eliminate quinolones and β-lactams (Poole, 2001), MexXY-OprM expels aminoglycosides (Hocquet et al., 2006), MexEF-OprN excretes quinolones (Moreira et al., 2004) and MexCD-OprJ gets rid of β-lactams (Okamoto et al., 2002). As for the OM permeability, the non-specific OM porin OprF is the dominant porin of *P. aeruginos*a and it has a low permeability for antibiotics (Sugawara et al., 2006) therefore characterizing *P. aeruginosa* with a permeability 10-100 fold lower than the one of *Escherichia coli* (*E. coli*) (Hancock & Brinkman, 2002). While OprD, present in low amounts, is a specific porin that allows the entry of carbapenems (H. Li et al., 2012). In addition, *P. aeruginosa*'s genome encodes an AmpC β-lactamase that inactivates β-lactam antibiotics (Wright, 2005). Lastly, *P. aeruginosa* lacks the large non-specific porins, OmpF or OmpC, found in other Gram-negative bacteria (Chevalier et al., 2017).

1.2.2. Adaptive resistance

Adaptive resistance is defined as a transient reversible state where bacteria reacts to an environmental stress by regulating the expression of resistance-associated genes allowing its survival (Sandoval-Motta & Aldana, 2016). Two major adaptive mechanisms can be observed in *P. aeruginosa* ; biofilm formation (previously described in 1.1.3.1) and the formation of antibiotic persisters (**Figure 6**). Antibiotic persistance is a phenomenon where a small population of bacteria survives a certain antibiotic while they are in a close-to-dormant state (Lewis, 2010). These cells do not divide, are metabolically inactive and present decreased intracellular ATP levels (Manuse et al., 2021) but can grow again once the antibiotic stress is removed (Maisonneuve & Gerdes, 2014; T. K. Wood et al., 2013).

1.2.3. Acquired resistance

In addition to its impressive intrinsic resistance, *P. aeruginosa* can become more resistant through mutations or by gaining resistance genes (López-Causapé et al., 2018). The acquired mutations could either affect efflux pumps by increasing their expression (such as MexAB-OprM and MexCD-OprJ) leading to a better elimination of the antibiotic or affect porins by inactivating them therefore reducing even more the OM permeability (Munita & Arias, 2016). For instance, inactivation of OprD porin confers an increased resistance to carbapenems (Fang et al., 2014). In addition, mutations causing an increased production of β-lactamase could also occur (Berrazeg et al., 2015). Lastly, mutations modifying antibiotics target take place, among them the mutations in ribosomal subunits (El'Garch et al., 2007), DNA binding proteins (Aldred et al., 2014) and lipopolysaccharides (LPS) (Owusu-Anim & Kwon, 2012). The other possibility is the acquisition of foreign genes, through horizontal gene transfer, carried by

plasmids, prophages and transposons (Breidenstein et al., 2011). The transferred material usually includes (multi)resistance cassettes and antibiotic inactivating enzymes such as broad-spectrum β-lactamases (Cavalcanti et al., 2015) and carbapenemases (J. Xiong et al., 2013) (**Figure 6**).

Subsequently, to face MDR pathogens such as *P. aeruginosa*, several alternative preventive and curative technologies are under current development. These strategies include antimicrobial peptides, phage therapy, nanoparticles for drug delivery, vaccines and monoclonal antibodies in addition to targeted gene editing using the CRISPR-cas system (Elfadadny et al., 2024; M. Hu & Chua, 2025).

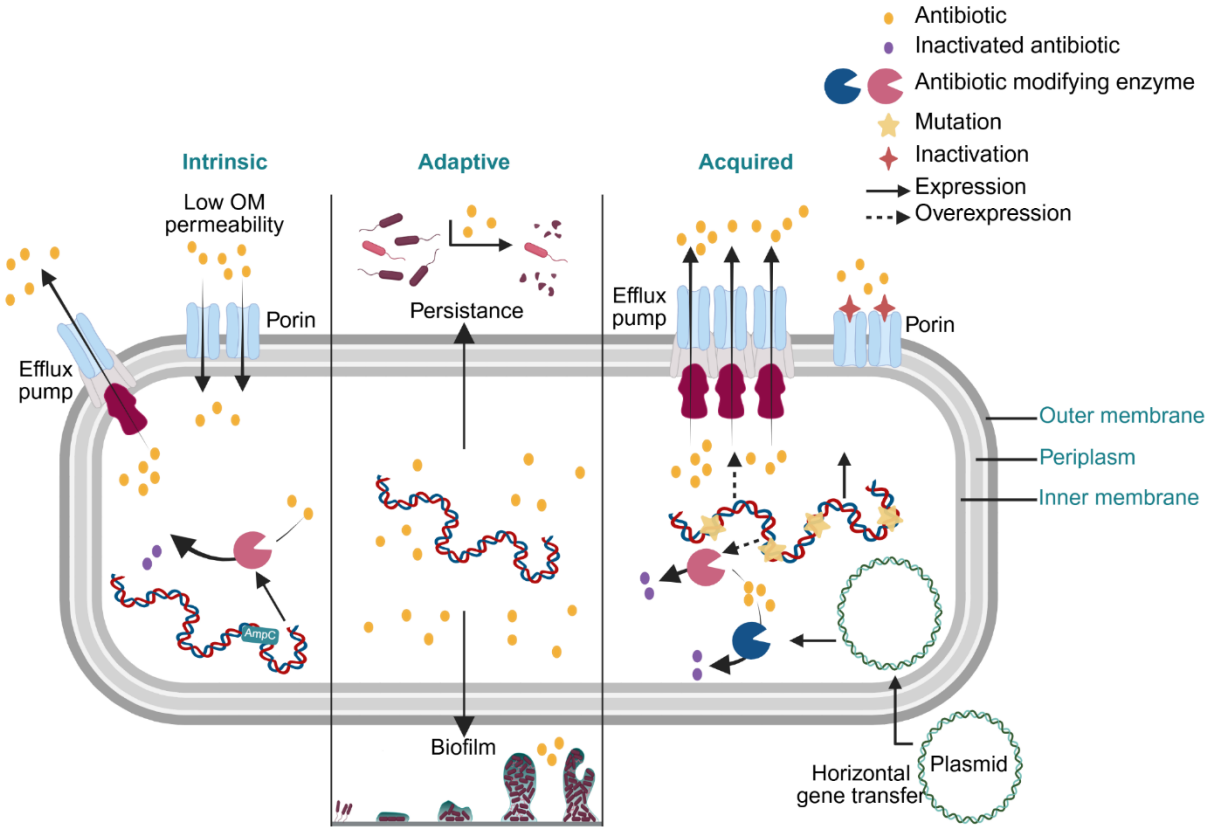

Figure 6. Antibiotic resistance mechanisms of *P. aeruginosa*.
P. aeruginosa resists to antibiotics through three mechanisms: intrinsic, adaptive and acquired. Intrinsic resistance includes naturally expressed efflux pumps and antibiotic modifying enzymes in addition to low outer membrane (OM) permeability (porins). The adaptive resistance is a transient state that includes biofilm formation and persisters. Acquired resistance is due to accumulated mutations in the genome of *P. aeruginosa* affecting the expression and functionality of efflux pumps, porins and antibiotic modifying enzymes in addition to the acquisition of exogenous antibiotic resistance genes through horizontal gene transfer. Inspired by (Qin et al., 2022). Made with Biorender.

1.3. *P. aeruginosa* virulence pathogenic toolbox overview

P. aeruginosa is equipped with an arsenal of virulence factors giving grounds to its pathogenic traits and its ability to survive not only the different environments that it is exposed to but also the diverse prokaryotic and eukaryotic enemies it encounters. This inventory consists of a collection of appendages, extracellular proteins and molecules whose production is under

complex signaling and regulatory networks. Here, *P. aeruginosa* resilience kit will be concisely described.

1.3.1. The lipopolysaccharide (LPS)

The LPS is a major component of the OM of Gram-negative bacteria (Pier, 2007). It is a protective glycolipid made of the hydrophobic lipid A, the core oligosaccharide and the O-antigen (Maldonado et al., 2016). The lipid A is endotoxic causing tissue damage, plays a role in biofilm formation and allows the adhesion of *P. aeruginosa* (Alshalchi & Anderson, 2015; J. S. Lam et al., 2011) and its recognition by host receptors (B. S. Park & Lee, 2013). The O-antigen, composed of sugar units, is highly variable and immunogenic (**Figure 7**) (Maldonado et al., 2016). As LPS protrudes out of the OM, it plays various roles in the interaction with the host : inhibition of phagocytosis and the deposition of the membrane attack complex (MAC – from the complement system), protects against oxidative stress and plays a role in motility and the attachment of bacteria to human epithelial cells (Huszczynski et al., 2019; Jamasbi & Taylor, 2010; King et al., 2009; Lindhout et al., 2009).

The LPS induces a robust (pro)inflammatory response *via* signaling through the toll-like receptor 4 (TLR4) and the CFTR (Huszczynski et al., 2019; Pier, 2007) leading to the production of several cytokines such as interleukin-1 (IL-1), IL-6, interferon gamma (IFN-γ) and tumor necrosis factor alpha TNF-α (Wieland et al., 2002).

1.3.2. Motility appendages

P. aeruginosa possesses one single polar flagellum constituted majorly of the polymerized flagellin (FliC), capped by the FliD protein, FlgE which is the hook at the base of the flagellum and two FlgKL proteins at the flagellum-hook junction in addition to other components crossing the inner and outer membranes (**Figure 7**) (Haiko & Westerlund-Wikström, 2013; Sampedro et al., 2015). The flagellum plays a crucial role in the swimming of *P. aeruginosa* in liquid and low viscosity media allowing it to move forward and backward (Sampedro et al., 2015). It permits the bacterium to reach the target cells, bind/adhere to epithelial cells and their extracellular matrix (Haiko & Westerlund-Wikström, 2013; Schwarzer et al., 2016). It is also involved in biofilm establishment (adhesion) and biofilm dispersal (motility) (Guttenplan & Kearns, 2013) and induces a TLR5-mediated immune response (Feuillet et al., 2006). Another motility accessory employed by *P. aeruginosa* is the T4P. It is a retractable filamentous appendage, made of thousands of molecules mainly major pilin (PilA) and other minor pilins at its tip (Jacobsen et al., 2020). Pili mediate adhesion, swarming and twitching motility allowing quick host colonization. This machinery ensures its function through consecutive cycles of extension, adhesion and retraction where PilA undergoes rounds of polymerization/depolymerization by ATPases (**Figure 7**) (Burrows, 2012). PilY1, at the tip,

recognizes host receptor and binds integrin, stabilizes the T4P and acts as a mechanosenor for *P. aeruginosa* (Heiniger et al., 2010; Johnson et al., 2011; Siryaporn et al., 2014). Pili also take part in biofilm formation and aggregation, protect against phagocytosis and induce host's immune response (inflammasome) (Arlehamn & Evans, 2011; Tan et al., 2014; Tolker-Nielsen, 2014). Interestingly, pili bind DNA hence its role in DNA transformation and the exchange of antibiotic resistance genes between bacteria (van Schaik et al., 2005).

1.3.3. *P. aeruginosa* secretion systems

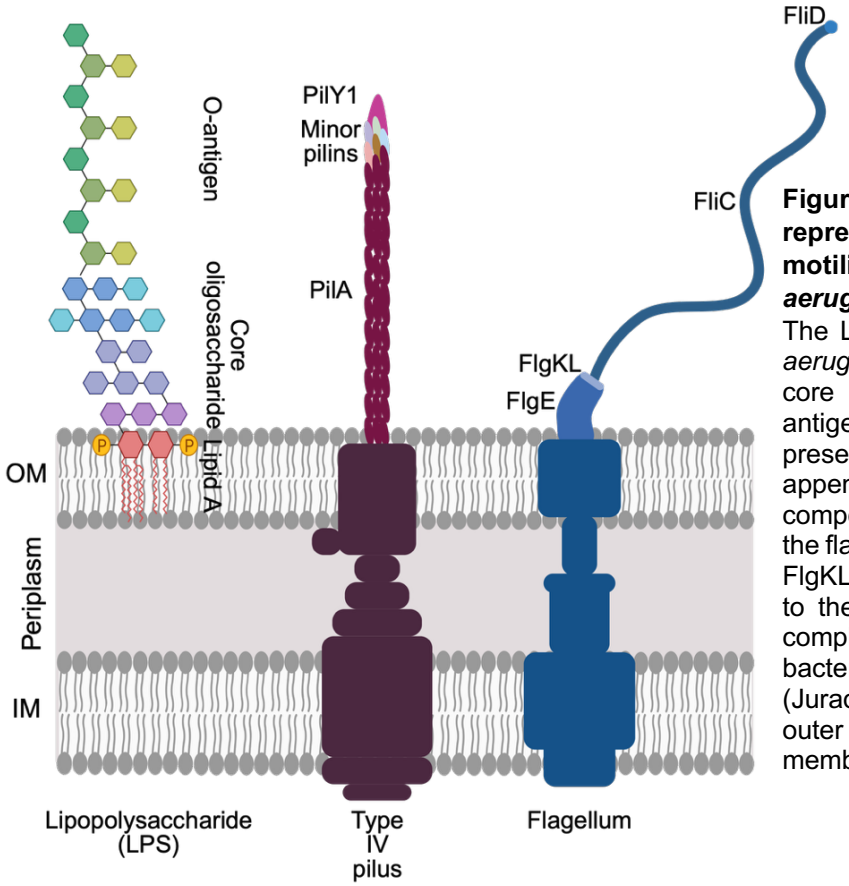

Figure 7. Schematic representation of the LPS and motility appendages of *P. aeruginosa*.
The LPS present in the OM of *P. aeruginosa* composed of lipid-A, a core oligosaccharide and the O-antigen (the LPS is schematically presented). The motility appendages Type IV pilus composed of pilin (Pil) proteins and the flagellum made of FliC, FliD and FlgKL and FlgE. Both are attached to the surface by their respective complexes embedded in the bacterial membranes. Inspired by (Jurado-Martín et al., 2021). OM: outer membrane, IM: inner membrane. Made with Biorender.

1.3.3.1. The Type 1 Secretion Systems (T1SS)

P. aeruginosa possesses two main T1SSs, the Apr and the Has systems which cross the inner and outer membrane and therefore secrete proteins in the extracellular medium (T. J. Smith et al., 2018). The Apr system uses three major proteins ; AprD, AprE and AprF and secretes the alkaline protease AprA and the AprX with an unknown function (**Figure 8**) (Delepelaire, 2004). AprA degrades complement system proteins and cytokines, hallmarks of the innate immune response, allowing bacterial escape (Hong & Ghebrehiwet, 1992; Laarman et al., 2012). It also breaks down the flagellin monomers inhibiting the pro-inflammatory cytokines released upon TLR5 activation (Bardoel et al., 2011). Whereas, the Has T1SS consists of HasD, HasE, HasF and the outer membrane factor (OMF) (Alav et al., 2021). It secretes a

heme-binding protein (hasAp) contributing to iron regulation (Cassat & Skaar, 2013). In addition, another T1SS effector was identified, TesG, able to supress the inflammatory response during chronic infections (K. Zhao et al., 2019).

1.3.3.2. The Type 2 Secretion Systems (T2SS)

The T2SS is a versatile tool secreting extracellularly and is composed of 11 to 16 proteins distributed at the inner and outer membrane and the periplasm (Bleves et al., 2010). *P. aeruginosa* harbors three T2SSs, the Xcp, the Hxc and the previously mentioned Txc systems. The Xcp secretes various exoproteins : exotoxin A (ETA), elastase A and B (LasA and LasB), phospholipase C (PLC), Lipase A and C (LipA and LipC) and protease IV (**Figure 8**) (Bleves et al., 2010). Las A and B lead to the destruction of the extracellular matrix of host cells, break down of cell-to-cell junctions and reduce host's immune response (phagocytosis and cytokines) (Bastaert et al., 2018; Nomura et al., 2014). The ETA is a toxic virulence factor that is internalized, after secretion, into host cell by interacting with the CD91 receptor. It inhibits proteins' synthesis by ADP ribosylating host elongation factor 2 (EF-2) eventually provoking programmed cell death (Bleves et al., 2010; Michalska & Wolf, 2015). Secreted PLC destroys host membranous phospholipids, and it could be hemolytic or not. It leads to host tissue damage, increased permeability and modulates inflammatory response in lungs (A. P. Barker et al., 2004; Z. Sun et al., 2014; Vasil et al., 1991). LipA is a major lipid-destroying enzyme that also plays a role in AMR within biofilm communities. The protease IV contributes to tissue damage and invasion in addition to immune system evasion. Besides, LapA and LapB of the Hxc system are alkaline phosphatases and are involved under phosphate-limiting conditions (Ball et al., 2012) and the Txc system releases the chitin-binding-protein E (CbpE) (Cadoret et al., 2014).

1.3.3.3. The Type 5 Secretion Systems (T5SS)

Together with the T1SS, they are considered the simplest secretion systems of *P. aeruginosa*. The T5SS includes the TPS (previously introduced in 1.1.2) and the autotransporters (AT) embedded in the OM (Bleves et al., 2010). The T5SS secretes EstA esterase, LepA large exoprotease and PlpD in addition to the extracellular adhesin CdrA through a TPS (**Figure 8**). The EstA esterase is anchored to the PM, has an esterase activity which was shown to be required for a higher production of rhamnolipids, promoting biofilm formation in addition to a role in adhesion and motility (Meuskens et al., 2019; Wilhelm et al., 2007) while PlpD is a phospholipase with a lipolytic activity *in vitro* (Salacha et al., 2010). Laslty, LepA regulates host immune response to bacterial infection by activating protease-activated receptors (PARs) (Kida et al., 2008, 2011). Despite this, many questions remain to be answered concerning the T5SS including structural studies, effectors identification and characterization and regulation.

1.3.3.4. The Type 6 Secretion Systems (T6SS)

The T6SSs of *P. aeruginosa* are encoded in three gene clusters termed T6SS hemolysin coregulated protein (Hcp) secretion islands H1 to H3, each with ~15-20 genes (Bleves et al., 2010; L. Chen et al., 2015). The T6SS is made of a baseplate structure, inner tube surrounded by a contractile sheath (ATP dependent) (Zoued et al., 2014). The H1, injecting only into bacterial cell, secretes the Tse1-3 effectors which can hydrolyze competing bacteria's cell wall giving *P. aeruginosa* an advantage in a competitive environment (Russell et al., 2011). Interestingly, H2 and H3 can target both prokaryotic and eukaryotic cells. Their phospholipases, PldA and PldB of H2 and H3 respectively (**Figure 8**), stimulate bacterial endocytosis allowing bacteria to invade host cells. In parallel, they have an antibacterial activity upon injection in the periplasm of prokaryotic cells (Jiang et al., 2014). Another H2 phospholipase, TplE, kills competitor bacteria and promotes autophagy in epithelial cells (Bleves, 2016). This dual interaction makes the T6SS particular as new functions and proteins are progressively unraveled paving the way to better understand the T6SS-induced pathogenicity.

This diversity greatly weakens the eukaryotic host defenses, yet the most important *P. aeruginosa* virulence factor is the Type 3 Secretion System (T3SS) will be detailed in chapter 2.

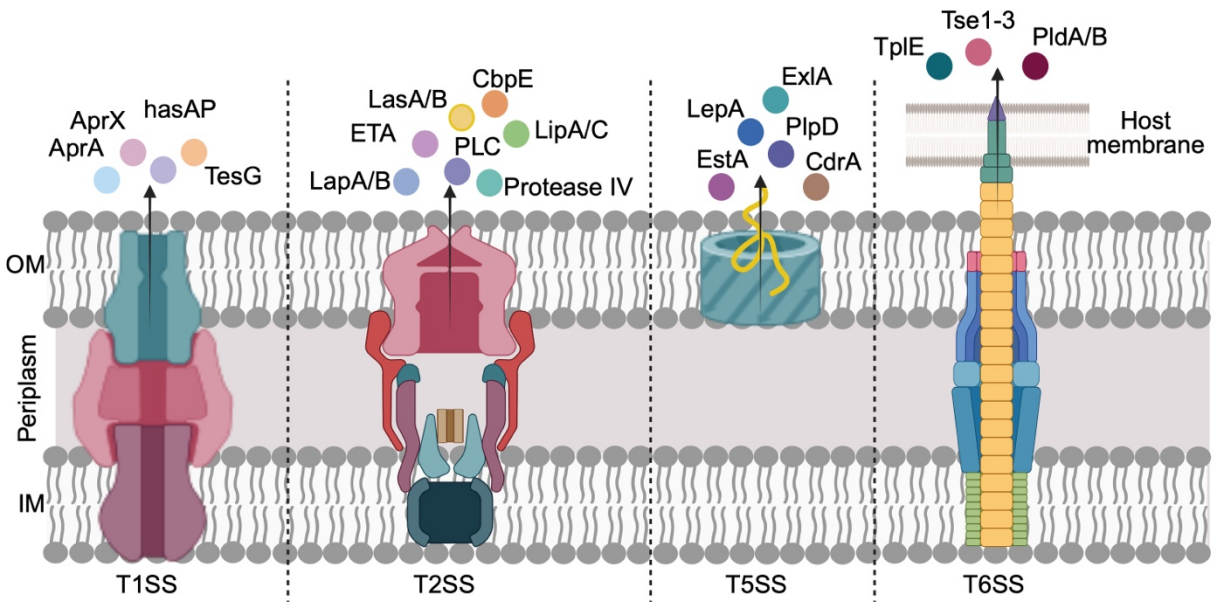

Figure 8. Schematic representation of the secretion systems of *P. aeruginosa* and their toxins. The secretion systems of *P. aeruginosa* include the T1SS, T2SS, T5SS and T6SS and are embedded in the membranes of the bacteria. The scheme focuses on the different toxins secreted by each system. The T6SS injects toxins directly into host cells cytoplasm (eukaryotic and/or prokaryotic host). The T3SS is not represented here. Inspired by (Qin et al., 2022). Made with Biorender.

Chapter 2 - The Type 3 Secretion System (T3SS)

The Type 3 Secretion System is a major virulence factor used by *P. aeruginosa*. As brought up in chapter 1, the T3SS exhibits 4 injected toxins, ExoS, ExoT, ExoY and ExoU, selectively present in the different strains. All isolates harbor the *exoT* gene, 89 % the *exoY* gene, 72 % the *exoS* gene and 28 % the *exoU* (Feltman et al., 2001) as the latter two are in most cases mutually exclusive (Ozer et al., 2019). ExoU and ExoS are more and more concomitantly detected in *P. aeruginosa* strains, for example in PA99 (ExoS/T/U) and ST463 (ExoS/U) (Feltman et al., 2001; Y. Hu et al., 2021) and several other stains (Nolasco-Romero et al., 2024). With such discoveries, we might witness in the upcoming years a new reclassification of *P. aeruginosa* isolates. For instance, ExoU is mostly associated with burns, wounds and bloodstream infections whereas ExoS in commonly found in isolates from CF patients (Feltman et al., 2001; Ozer et al., 2019). ExoU infections are more cytotoxic than ExoS infections and are associated with a higher morbidity (Ozer et al., 2019; Pena et al., 2015).

The T3SS is composed of a needle that connects *P. aeuginosa* to the host, translocators at the tip of the needle, effectors and chaperones all tightly regulated through a complex network (Hauser et al., 1998).

2.1. The apparatus and translocators of the T3SS: components and assembly

The injectisome is divided into 3 structural identities each with its specialized proteins: cytoplasmic, membranous and extracellular.

In the cytoplasm lies the cytoplasmic ring (c-ring) and the ATPase complex together termed the sorting platform. The c-ring is made of PscQ that encases the sorting platform (W. Deng et al., 2017) while the ATPase complex includes 4 proteins : the ATPase PscN, the cofactor PscK and the regulators PscO and PscL (**Figure 9 B**). PscO links PscN to the export apparatus located in the membrane while PscL is a negative regulator of PscN (W. Deng et al., 2017; Halder et al., 2019). The sorting platform plays a role in the recruitment of molecules (components or effectors) to be secreted by the T3SS (Notti & Stebbins, 2016) and the regulation of a correct order of engagement is also ensured by the switch proteins PopN, PscP and PscU (W. Deng et al., 2017; Journet et al., 2003; Ngo et al., 2020).

The membranous compartment consists of the basal body that is composed first of the outer and inner rings made of the PscC protein and the PscJ and PscD proteins respectively (Notti & Stebbins, 2016). A channel is created between the two rings and is coated by PscI protein forming the inner rod linking the inner ring to the needle (PscF) (W. Deng et al., 2017). Lastly, the basal body also includes an export apparatus that interacts with the cytoplasmic ring and is present in the inner membrane (IM) (W. Deng et al., 2017; Wagner et al., 2010). It is composed of PcrD, PscU and PscRST: PcrD connects the secretion pore to the ATPase

complex, PscU regulates the secretion switch and the other 3 proteins, PscR, PscS and PscT, are in the IM (**Figure 9 B**) (W. Deng et al., 2017; Dietsche et al., 2016).

Lastly, the needle protrudes from the system and is therefore bridging PscI (periplasm) with the translocation pore in the host's membrane. The needle is made of units of the PscF protein assembled into a right-handed helicoidal structure with about 11 subunits per two turns (Hauser, 2009; Pastor et al., 2005). The needle is around 6-7 nm diameter, 60-80 nm length with an inner diameter of 2-2.5 nm (Lombardi et al., 2019; Pastor et al., 2005). At the tip of the needle comes PcrV acting as a linker, for PopB and PopD, between the needle and the host membrane (Goure et al., 2004). PopB and PopD lead to a pore formation of 1-4 nm and subsequent effectors translocation into host cell cytoplasm (**Figure 9 B**) (F. B. Romano et al., 2011).

A

Common	*P. aeruginosa*
SctN	PscN
SctL	PscL
SctO	PscO
SctQ	PscQ
SctV	PcrD
SctK	PscK
SctU	PscU
SctRST	PscRST
SctJ	PscJ
SctD	PscD
SctI	PscI
SctC	PscC
SctF	PscF
SctA	PcrV
SctP	PscP
SctE/SctB	PopB/PopD

B

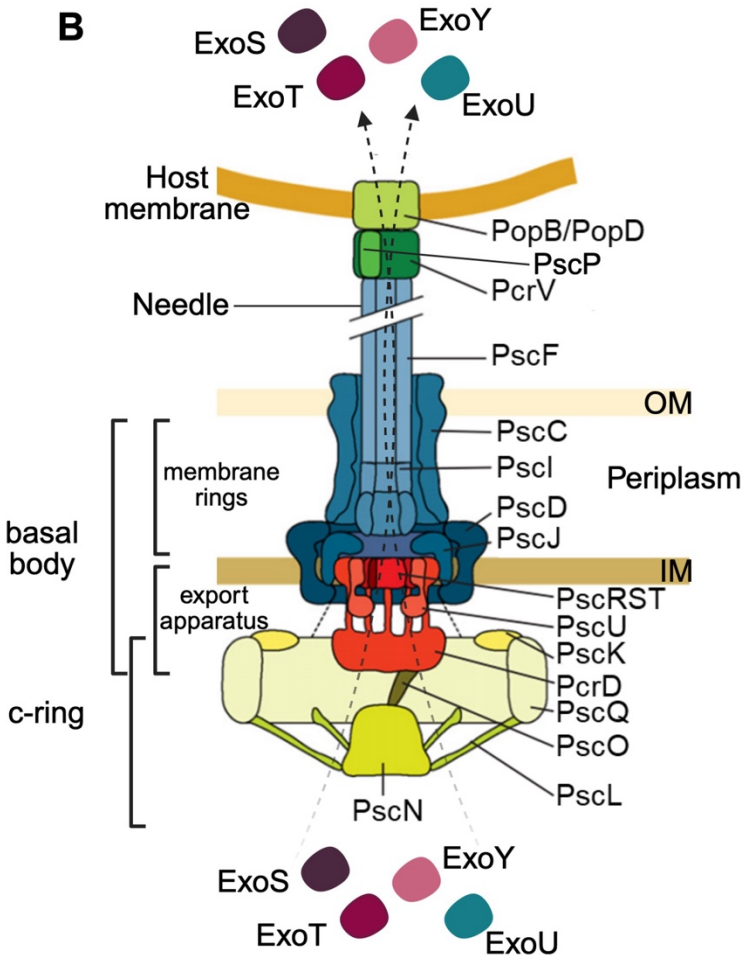

Figure 9. The T3SS of *P. aeruginosa* and its components
(A). Nomenclature of the T3SS components that is commonly used and the terms adopted for *P. aeruginosa* T3SS proteins. From (Ngo et al., 2020). **(B)**. The organization of the T3SS of *P. aeruginosa*, composed of cytoplasmic, membranous and extracellular components leading to the translocation of T3SS toxins ExoS, ExoT, ExoY and ExoU in host cell cytoplasm. Adapted from (Diepold & Wagner, 2014).

2.2. Regulation of the T3SS expression

Most of the T3SS genes are encoded in five operons clustered in the genome of *P. aeruginosa* which expression is modulated by the ExsA transcriptional regulator (Hauser, 2009). ExsA is located within the *exsCEBA* operon and triggers its own expression in addition to the one of the T3SS operons (Hauser, 2009; Yahr & Wolfgang, 2006) (**Figure 10 A**). Three other proteins, ExsC, ExsE and ExsD are involved in the ExsA-dependent transcriptional modulation of the T3SS. In the absence of any stimulation, ExsD blocks the activity of ExsA by binding it in a 1 :1 ratio (Thibault et al., 2009). ExsC has a naturally high affinity for ExsE to which it binds as its chaperone within the bacteria. Upon T3SS stimulation by low Ca^{2+} levels or a contact with the eukaryotic host cell, ExsE is released from the ExsE-ExsC complex and is secreted through the T3SS needle. In this case, ExsC becomes free and interferes with the ExsA-ExsD interaction, liberating ExsA and allowing the ExsA –dependent transcription of the T3SS (**Figure 10 B**) (Hauser, 2009; Rietsch et al., 2005; Yahr & Wolfgang, 2006).

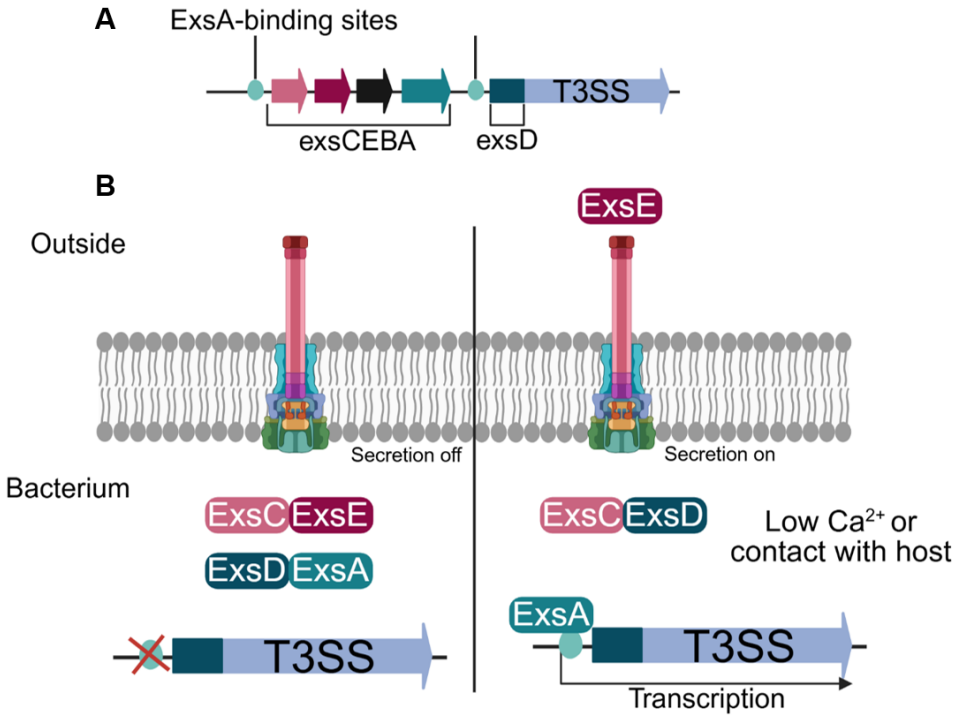

Figure 10. Regulation of the expression of the T3SS.
(A). Genetic organization of the ExsCEBA operon that controls the expression of the T3SS in an ExsA-dependent manner. **(B)**. Schematic representation of the induction of the expression of the T3SS genes upon low intracellular Ca^{2+} levels or contact with host cells. In the off state, ExsA is confiscated by ExsD and EsxC interacts with ExsE. In the on state, ExsE is secreted through the T3SS and ExsC interferes with the ExsD-ExsA interaction freeing ExsA that is now able to induce the transcription of the T3SS. The T3SS gens are organized in five operons dependent on ExsA but schematically only two are shown. Inspired by (Yahr & Wolfgang, 2006). Made with Biorender.

2.3. The T3SS Toxins

The T3SS toxins ExoS, ExoT and ExoU have a common architecture based on four parts: secretion signal, chaperone binding domain (CBD), membrane localization domain (MLD), in addition to own specific domains for each toxin (**Figure 11**). On the other hand, ExoY has a distinct architecture, it does not harbor neither a CBD nor a MLD (Horna & Ruiz, 2021). The CBD allows the interaction of the toxin with its respective chaperone (Parsot, 2003). Chaperones supervise the correct recruitment of the effectors to the T3SS where the ATPase ensures their unfolding, a state required for efficient secretion (Akeda & Galán, 2005; Galle et al., 2012). It is to note that the T3SS components, other than the effectors, also have their cognate chaperones. Such as PcrH for PopB and PopD, PcrG for PcrV and PscE-PscG for PscF (Allmond et al., 2003; Nanao et al., 2003; Quinaud et al., 2005). In addition to the main T3SS toxins, in 2015, novel T3SS effectors were identified, PemA and PemB but they were not cytotoxic and yet their function was undetermined (Burstein et al., 2015). Recently, PemB was shown to enhance the toxicity of the PA14 strain in *Caenorhabditis elegans* infection model (Zelikman et al., 2024). Until today, these Pem proteins are not widely recognized as “effectors” and are not actively studied.

2.3.1. The exotoxin S (ExoS)

The *exoS* gene encodes the 48.3 kDa ExoS toxin of 453 amino acids. It interacts with its chaperone SpcS prior to secretion (Shen et al., 2008). ExoS is a bifunctional T3SS toxin with the N-terminal region (aa 96 to 233) harboring a GTPase activating protein (GAP) domain (**Figure 11**). The GAP domain targets host’s small GTPases such as Rac, Rho and Cdc42 leading to the disturbance of adhesion between cells and the disruption of the actin cytoskeleton. The GTPases are held inactive when bound to GDP promoting actin depolymerization, cell rounding and eventual cell death (Hauser, 2009; Huber et al., 2014; Tukaye et al., 2013). As for the C-terminal region of ExoS (aa 233 to 453), it harbors an adenosine diphosphate ribosyl transferase (ADPRT) activity. This domain also targets small GTPases such as Rab, Rac, Ras and Cdc42 in addition to the ERM proteins (ezrim, radixin and moesin) which link the PM to the actin (Jia et al., 2006; Maresso et al., 2007). The ADPRT region was found to interact with host 14-3-3 proteins as chaperones preventing it from auto-aggregating, making this interaction essential for ExoS’ correct activation and functioning (Ottmann et al., 2007). The ADPRT activtity of ExoS has valuable consequence on host cells : retraction and loss of adhesion, decreased proliferation, indcution of cell death by apoptosis, inhibition of DNA synthesis and vesicular trafficking, stimulation of the inflammatory response and inhibition of phagocytosis (Q. Deng & Barbieri, 2008; Fraylick et al., 2001; Hauser, 2009;

Rangel et al., 2014). Overall, ExoS facilitates bacterial persistance and dissemination within the host and contributes to host tissue damage.

The trafficking of ExoS within host cell and its interaction with the vesicular trafficking will be detailed in section 3.2.4.1.

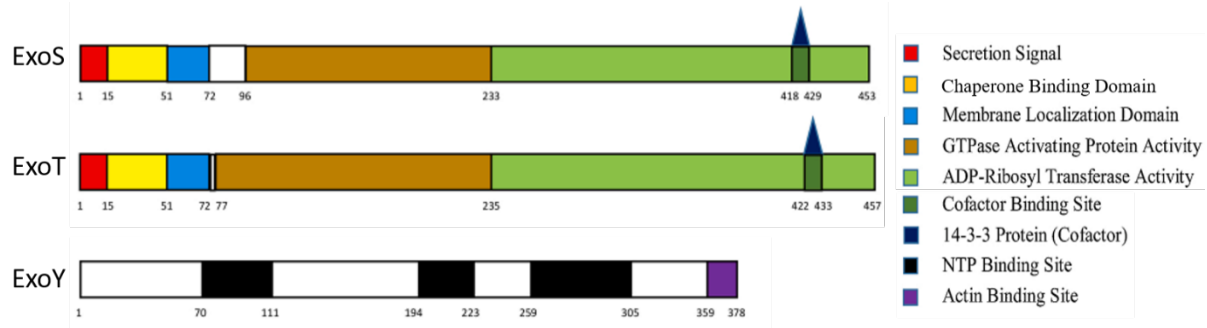

Figure 11. Domains of the ExoS, ExoT and ExoY toxins of the T3SS of *P. aeruginosa*.
The ExoS and ExoT toxins have similar domains whereas ExoY is quite distinct and is devoid from a secretion signal. ExoS and ExoT are bifunctional with the N-terminal region harboring a GTPase activating protein (GAP) domain and the C-terminal region of ExoS with an adenosine diphosphate ribosyl transferase (ADPRT) activity and ExoY is a nucleotidyl cyclase. The triangles denote the 14-3-3 cofactor bound at its site in ExoS and ExoT. Adapted from (Horna & Ruiz, 2021).

2.3.2. The exotoxin T (ExoT)

ExoT is a 48.5 kDa (457 aa) T3SS toxin, is 76 % similar to ExoS and has both domains, the GAP (aa 78 to 235) and ADPRT (aa 235 to 457) regions (**Figure 11**) (Winsor et al., 2016). It uses the same bacterial chaperone SpcS for secretion and the host chaperone 14-3-3 proteins for its ADPRT activity (Karlberg et al., 2018; Shen et al., 2008). The GAP domain similarly targets GTPases affecting actin organization leading to cell rounding and induces apotptosis (Garrity-Ryan et al., 2000; Hauser, 2009). Whereas the ADPRT domain targets the Crk proteins (CT10 Regulator of Kinase) Crk1 and 2 in addition to a phosphoglycerate kinase. The ADP rybosylation of these proteins leads to an atypical cell death known as anoikis (type of apoptosis). In fact, the modification of Crk disrupts focal adhesion sites and interferes with integrin-mediated survival signaling pathway promoting host cell death (J. Sun & Barbieri, 2003; S. Wood et al., 2015).

2.3.3. The exotoxin Y (ExoY)

ExoY is a protein of 41.7 kDa composed of 378 aa and is a T3SS toxin which chaperone has still not been identified (Yahr et al., 1998; Khanppnavar et al., 2020). ExoY possesses a nucleotidyl cyclase activity (**Figure 11**) which, 6 hours following its injection, leads to the production of high levels of c-AMP, c-GMP and cUMP (Morrow et al., 2015). Distinctively from ExoS and ExoT, ExoY is activated by host filamentous actin (f-actin) through ExoY's C-terminal region altering actin's turnover and therefore promoting cell rounding (Belyy et al., 2016, 2018). The high level of cAMP compromises the endothelial barrier by activating the kinase A

therefore phosphorylating tau, a protein associated to microtubules which induces mircrotubules disassembly (Ochoa et al., 2012; Morrow et al., 2017). In addition, elevated levels of cNMPs modulate the host's immune response in favor of *P. aeruginosa* by the inhibition of the MAPK regulator TAK1 (TGF-β activated kinase 1). Consequently, the NF-κB pathway and the production of IL-1β are supressed advocating for *P. aeruginosa*'s escape from the host's immune response and increases the severity of the infection (C. He et al., 2017).

2.3.4. The exotoxin U (ExoU)

The strains expressing the ExoU toxin are the most cytotoxic for the eukaryotic cells and are associated with severe lesions in addition to a higher antibiotic resistance making it one of the most studied and the most potent T3SS effector (Sawa et al., 2014; Akter et al., 2025).

2.3.4.1. The domains of ExoU

ExoU is a 73.9 kDa protein of 687 aa encoded within the pathogenicity island 2 (PAPI-2) of *P. aeruginosa* followed by its chaperone, SpcU of 15 kDa (137 aa) (J. He et al., 2004). ExoU is composed of different domains and begins with a secretion signal at the first 15 aa at its N-terminus which is present in all T3SS effectors (Hauser, 2009). It is followed by the CBD which is necessary for the interaction with SpcU and is located between the aa 52 to 100 of ExoU. The CBD is followed by the PLP (patatin-like phosphatase) domain (106-471) responsible for the phospholipase A2 (PLA2) activity of ExoU and harboring the catalytic dyad serine 142 (S142) and aspartate 344 (D344) residues (Phillips et al., 2003; Sato et al., 2003) (**Figure 13 AB**). PLA2s hydrolyze phospholipids at position sn-2 thereby generating a free fatty acid such as the arachidonic acid and a lysophospholipid (**Figure 12**) (Lambeau & Gelb, 2008). The catalytic domain is flanked by the two bridging subdomains, the N-terminal of 28 aa (73 to 100) and the C-terminal one being of 100 aa (480 to 580) (Gendrin et al., 2012). Then comes the MLD from residue 588 to 687 (**Figure 13 BC**) which is essential for ExoU to localize to its target, the PM (Rabin et al., 2006; Veesenmeyer et al., 2010). The MLD was revealed to be a four-helical bundle (Gendrin et al., 2012). It was proposed that the C-terminal bridging domain plays a role in bringing closer the phospholipase domain and the MLD, and therefore the PM, promoting the activity of ExoU (Gendrin et al., 2012).

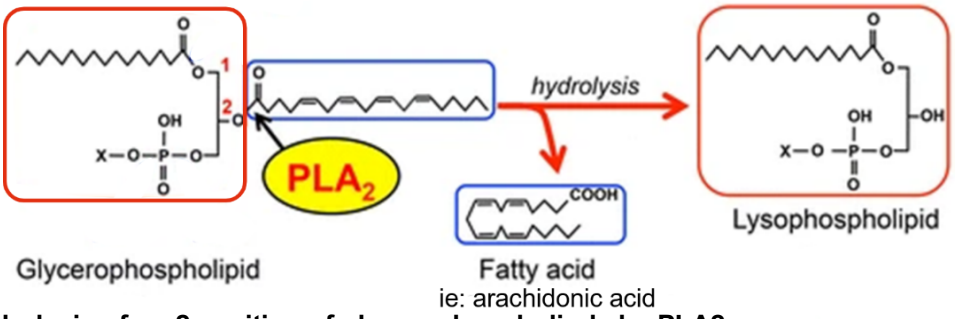

Figure 12. Hydrolysis of sn-2 position of glycerophospholipds by PLA2.
The hydrolysis of sn-2 position by PLA2 generates a fatty and a lysophospholipid. Adapted from (Sato et al., 2016)..

2.3.4.2. The ExoU-SpcU complex prior to secretion

Within *P. aeruginosa*, ExoU is held inactive by SpcU. The structure of ExoU with SpcU in a 1:1 complex has been solved and SpcU interacts with other ExoU regions (**Figure 13 C**) (Gendrin et al., 2012; Halavaty et al., 2012). Precisely, the ExoU-SpcU structure portrays a flexible region blocking/closing the active site of ExoU where the flexible region includes the D344 residue. To allow the activation of ExoU, this flexible region must be repositioned to allow the substrate to access the active site (Gendrin et al., 2012). Upon T3SS activation, the ExoU-SpcU complex is recruited to the sorting platform where the ATPase PscN hydrolyzes ATP,

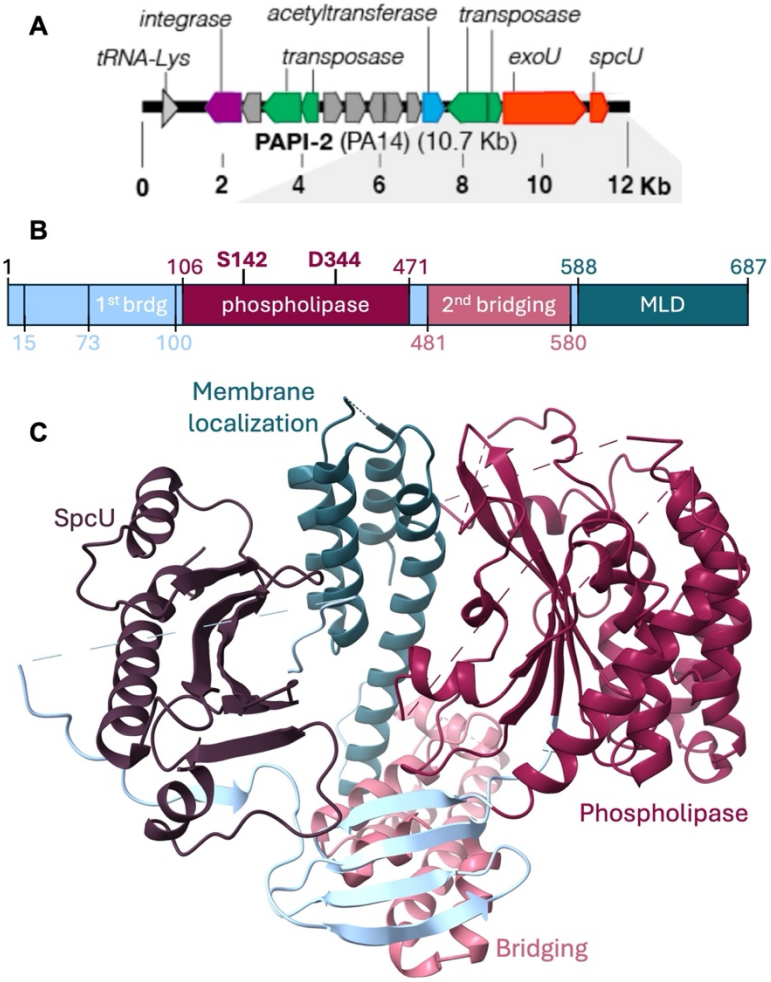

Figure 13. Genetic and protein organization of ExoU.
(A). Schematic representation of the *Pseudomonas aeruginosa* pathogenicity island 2 (PAPI-2) containing the *exoU* gene followed by its chaperone *spcU*. From (Sawa et al., 2016). **(B)**. The domains of ExoU with the catalytic residues S142 and D344 in the phospholipase domain. **(C)**. The crystal structure of ExoU with its bacterial chaperone SpcU in 1:1 complex ratio. Four ExoU domains are represented each with its respective color. Adapted from (Gendrin et al., 2012).

liberating ExoU from SpcU. ExoU is unfolded, secreted through the T3SS needle and translocated into host cells cytoplasm where it is refolded and activated (Akeda & Galán, 2005; Halder et al., 2019).

2.3.4.3. Host factors required for the phospholipase activity of ExoU

2.3.4.3.1. The phosphatidylinositol-4,5-bisphosphate PI(4,5)P_2

PI(4,5)P_2 is a negatively charged lipid mainly present in the inner leaflet of the PM of eukaryotic cells and is concentrated in lipid rafts yet it constitutes 1-2 % of the total membrane lipid composition (**Figure 14**) (McLaughlin et al., 2002; McLaughlin & Murray, 2005). When hydrolyzed by phopspholipase C, it generates two secondary messengers; diacylglycerol (DAG) and inositol (1,4,5)-trisphosphate (IP_3) affecting signaling pathways (Berridge, 1987). PI(4,5)P_2 is involved in a variety of cellular events and processes related to the PM including cell adhesion, actin reorganization, ion channels regulation, phagocytosis, exocytosis and endocytosis (reviewed in (Katan & Cockcroft, 2020).

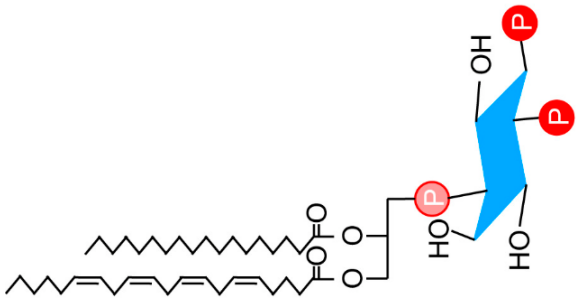

Figure 14. Structure of phosphatidylinositol-4,5-bisphosphate PI(4,5)P2.
Schematic representation of the PI(4,5)P2 with phosphate groups at position 4 and 5 in addition to two fatty acid chains. From (Harraz et al., 2020)

Membranous fractions promoted a strong ExoU phospholipase activity and specifically when PI(4,5)P_2 is present. It was found to be the sole lipid activator of the ExoU phospholipase activity *in vitro* even at concentrations lower than the physiological concentrations (Stirling et al., 2006; Tyson & Hauser, 2013a). In addition to the activation of ExoU, PI(4,5)P_2 was shown to interact with the C-terminal domain, the MLD, of ExoU in order to localize it to the PM (Rabin et al., 2006). This was observed by mutating residues between 550 to 687 aa of ExoU. Specifically, residues lysine 679, arginine 682 and asparagine 683 (Lys-679, Arg-682 and Asn-683) were responsible for this interaction (**Figure 15 A**) as when deleted, the association between ExoU and the PI(4,5)P_2, therefore with the PM, is abrogated (Rabin et al., 2006; Gendrin et al., 2012; Tyson et al., 2015). These residues form a polar basic « backbone » able to interact with negatively charged lipids (Gendrin et al., 2012). In addition, structural analyses pointed towards residues Arg-620, Gln-623 and Arg-661, present in loops L1 and L3 **(Figure 15 B**) of the MLD that could strengthen and maintain the interaction between ExoU and the inner leaflet negatively charged lipids (Veesenmeyer et al., 2010; Tyson & Hauser, 2013a; Springer et al., 2019). It is to note that ExoU oligomerizes, 6 to 8 monomers, at the level of the

PM in a PI(4,5)P_2 and MLD-dependent manner. This oligomerization enhances the phospholipase activity of the individual ExoU monomers (A. Zhang et al., 2018).

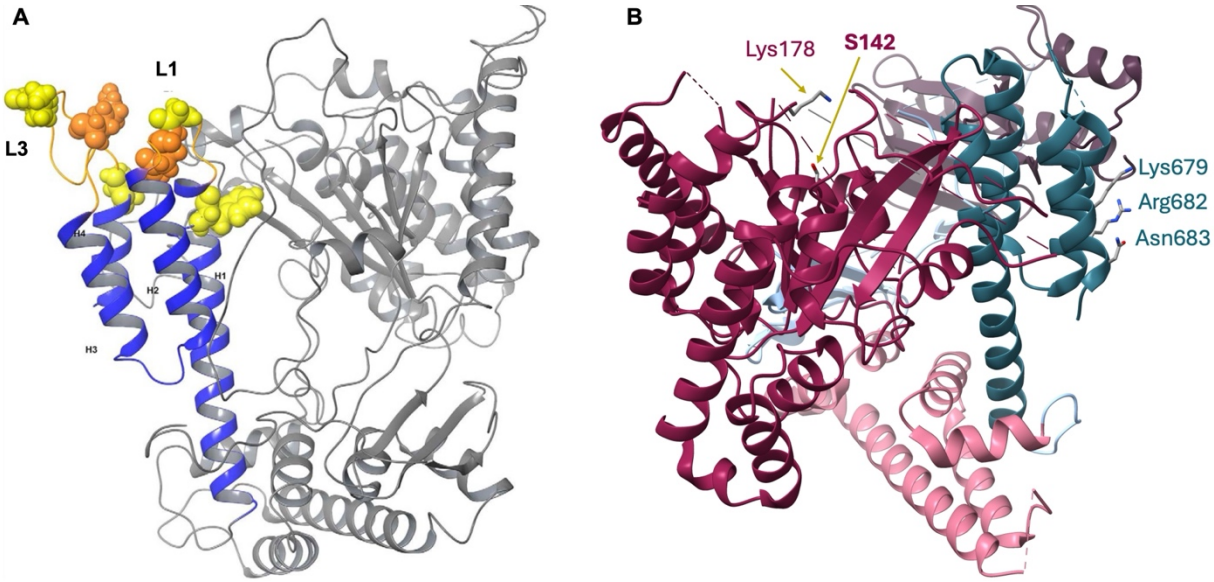

Figure 15. The MLD domain of ExoU that interacts with PI(4,5)P2 at the host PM.
(A). Model of the loops (L1 and L3) that strengthen the interaction between the MLD and the PI(4,5)P2 at the level of the PM. L1 and L3 are shown in orange and residues Gln-623 and Arg-661 in L1 and L3 respectively are in orange spheres. The bridging and catalytic domains are in gray and the four-helix bundle (MLD) is in purple. From (Springer et al., 2019). **(B)**. The structure of ExoU pinpointing the catalytic residues in the phospholipase domain (cyan) and the critical residues for the interaction with PI(4,5)P2 at the PM within the MLD (red). Adapted from (Gendrin et al., 2012).

2.3.4.3.2. Ubiquitin

In addition to the PI(4,5)P_2, the presence of ubiquitin alone can stimulate the activity of ExoU whereas the optimal activity is achieved when both synergic cofactors are provided. In addition, ExoU was found to be ubiquitinated upon injection in the host cytoplasm (Stirling et al., 2006; Tyson & Hauser, 2013b; Tyson et al., 2015; Anderson et al., 2011). It was proposed that ExoU interacts non-covalently with ubiquitin through its bridging domain, particularly the Thr-519, Thr-522, Val-523 and Val-526 residues that could interact with the hydrophobic part of ubiquitin (Tessmer et al., 2018). The mutation of these residues abrogates the ExoU phospholipase activity in a cellular model and when expressed in *E. coli* with monoubiquitin (Anderson et al., 2013; Tessmer et al., 2018) highlighting the importance of the ubiquitination at these specific residues.

In addition, ExoU was covalently ubiquitinated by two ubiquitin molecules at the Lys-178 of ExoU (Stirling et al., 2006). Interestingly, the mutation of Lys178 to Arg-178 has no effect whatsoever neither on the lipase activity of ExoU nor on its PM association (Stirling et al., 2006; Gendrin et al., 2012). Yet, it does modify the cytoplasmic localization of ExoU during its trafficking within the eukaryotic host (Gendrin et al., 2012). The journey of ExoU upon injection in the host cytoplasm trafficking to its PM target will be thoroughly described in section 3.2.4.2.

With these two host cofactors, ExoU is at its maximal necrotic potential. It was proposed that the L1 and L3 loops first deeply anchor ExoU in the PM. The MLD is then extended away from the catalytic site while the bridging domain is moved in a way stabilizing the interaction ExoU-$PI(4,5)P_2$. Lastly, a conformational change occurs within the catalytic domain where the previously mentioned flexible loop is rearranged, opening up the catalytic site towards the lipids of the inner leaflet of the PM (**Figure 16**). In parallel, the most pronounced conformational modification of ExoU occurs in the presence of ubiquitin (Tessmer et al., 2017; Feix et al., 2019). Our knowledge is still limited when it comes to the kinetics of the ubiquitination, its concrete role and the structural modifications occurring with ExoU, requiring therefore further studies.

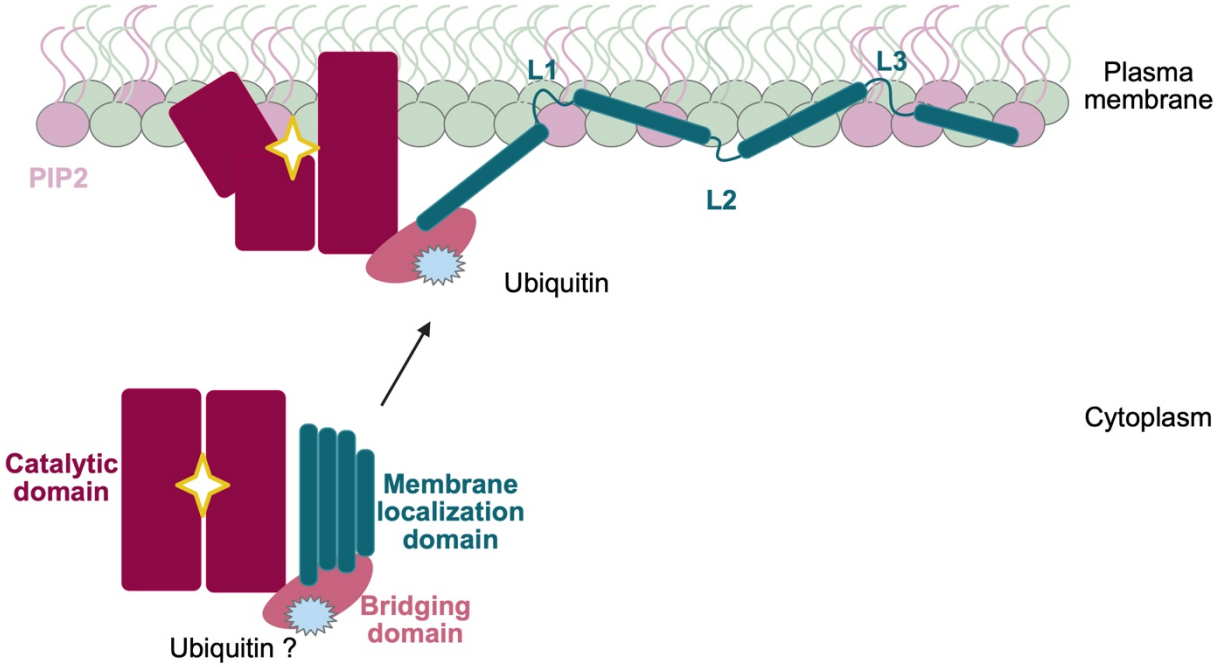

Figure 16. Conformational rearrangements occurring to allow the phospholipase activity of ExoU. The catalytic domain with the catalytic site (star), the bridging domain and the four-helix bundle of the MLD are presented. Upon interaction of the MLD with the PM and ubiquitination of the bridging domain, structural modifications occur to allow the catalytic site of ExoU to interact with its substrate leading to the activation of ExoU. According to (Tessmer et al., 2017). Made with Biorender.

2.4. Aftermath of ExoU injection and activity on the eukaryotic cell

The presence of ExoU within host cells induces an inflammatory response leading to the production of IL-8. This is possible either by the activation of the MAPK-JNK signaling pathway (Cuzick et al., 2006) or through the synthesis of ecosanoids such as prostaglandins (PGE2 and PGI2) using the arachidonic acid provided by the PLA2 activity. These prostaglandins stimulate the inflammatory response and increase the production of IL-6 and IL-8 (J.-S. Cho et al., 2014; Kawahara et al., 2015). The IL-8 chemokine attracts neutrophils to the site where *P. aeruginosa* is localized (Pazos et al., 2017). At this stage, *P. aeruginosa* is able to kill neutrophils and other phagocytic cells that come its way through the ExoU activity (Diaz &

Hauser, 2010). In addition, ExoU inhibits the generation of pro-inflammatory cytokines such as IL-1β (Sutterwala et al., 2007).

In parallel, the PLA2 activity of ExoU compromises the host's PM by hydrolyzing the $PI(4,5)P_2$ (Phillips et al., 2003) and consequently, once activated, a variety of phospholipids among them phosphatidylcholine and phosphatidylethanolamine (Sato et al., 2003). The destruction of membranous $PI(4,5)P_2$ leads to cytoskeletal breakdown, loss of PM integrity, loss of focal adhesion, cell rounding and retraction prior to cell death (Sato & Frank, 2014). The ExoU activity causes severe lesions notably in epithelial cells, breaching the epithelial barrier, promoting *P. aeruginosa*'s dissemination throughout the systemic circulation and reaching other organs (Kurahashi et al., 1999; Phillips et al., 2003; Shaver & Hauser, 2004). Overall, ExoU induces the general inflammatory response, yet it can overcome the local innate immune response and cause remarkable tissue damage to the host favoring the survival of *P. aeruginosa*. Combining these consequences, the end game of the ExoU intoxication is cell death by necrosis.

Chapter 3 – How bacteria exploit and manipulate host trafficking pathways

Over the years, many studies aimed to decipher the molecular mechanisms governing bacterial infections affecting humans focusing on cellular responses, cell death, signaling pathways, bacterial evasion of the host's immune system, associated virulence factors and the hijacking of cellular machineries. All works contributed to better understand these interactions on the molecular level and pave the way to fight infections.

In this PhD project, we focused on the trafficking of bacterial toxins within the host and the hijacking of the corresponding cellular machineries. Therefore, the upcoming section will highlight a variety of bacterial pathogens and their toxins/effectors diversely manipulating membrane trafficking pathways. We begin with an overview on the different branches within trafficking pathways and their general markers and in the second part, specific bacterial and eukaryotic factors involved in a certain interaction will be gradually introduced.

It is to note that this chapter of the manuscript is meant to be further modified and submitted as a review.

3.1 Main eukaryotic intracellular trafficking pathways

Intracellular trafficking pathways are essential for the functioning and survival of all cells (Gimeno-Agud et al., 2025). They are involved in many cellular processes such as homeostasis, communication and the recycling and degradation of their content (Buratta et al., 2020; Gimeno-Agud et al., 2025). Trafficking pathways include classical membrane trafficking (endocytosis and exocytosis) in addition to a distinct process known as autophagic pathway (Bento et al., 2013; Gimeno-Agud et al., 2025). The three paths are distinguished by their own molecular machinery yet they remain inevitably intertwined as they communicate through vesicular transport mediated by the cytoskeletal filaments and motor proteins (Münz, 2017; Papandreou & Tavernarakis, 2020; Gimeno-Agud et al., 2025; Phelps et al., 2003). Vesicles are spherical membranous structures that transport cellular products between the different cellular compartments and to the extracellular milieu.

The endoplasmic reticulum (ER) and the Golgi apparatus are the two main membrane trafficking concerned organelles (Gimeno-Agud et al., 2025). The ER is the largest cell organelle with a major role in proteins synthesis, folding and trafficking, lipid metabolism and calcium signaling (Rapoport, 2007; Clapham, 2007; Braakman & Hebert, 2013; Fagone & Jackowski, 2009). As for the Golgi apparatus, it exchanges proteins and lipids with the ER where they are modified and sorted before being transported to their final destination (Huang & Wang, 2017; K. Park et al., 2021). The trafficking from the ER to the Golgi is called anterograde trafficking while the opposite process is known as retrograde (García-Mata et al., 2003; Gimeno-Agud et al., 2025; Miller et al., 2003).

3.1.1 Exocytosis

Exocytosis is the cellular process by which vesicles are transported within the cells towards the plasma membrane or the extracellular milieu (Rizo & Südhof, 2012a; Weigel et al., 2021). It includes the delivery of lipids, proteins or molecules such as hormones and neurotransmitters (Gillon et al., 2012; J. J. Park & Loh, 2008; Rizo & Südhof, 2012b) herein referred to as cargoes. Exocytosis begins at the ER, specifically at the ER exit sites (ERES) with the help of COP-II coatomer complex that allows vesicles' budding. From there, they localize to the tubular vesicular ER-Golgi intermediate compartment (ERGIC). Vesicles are then transported to the Golgi apparatus where they undergo post-translational modifications passing successively through the continuous cisternae of the Golgi system: cis-Golgi network (CGN), medial and reaching the trans-Golgi network (TGN) where they are released towards their destination such as other organelles, plasma membrane (PM) or extracellular environment. Proteins could travel also in a retrograde path from the Golgi to the ER in a COP-I coatomer complex-dependent manner (**Figure 17**) (Mironov et al., 2003; Mellman & Nelson, 2008; Weigel et al., 2021).

3.1.2 Endocytosis

Endocytosis is a process by which cells sense their outside environment and incorporate exogenous molecules or surface proteins (cargoes) into membranous compartments within cell cytoplasm (Blanco & Blanco, 2017; Mellman & Nelson, 2008; Sigismund et al., 2021). It usually occurs through PM invagination and cleavage leading to the formation of vesicles that fuse with early endosomes (EE) (Poteryaev et al., 2010; Salzman & Maxfield, 1988). Several internalization mechanisms have been identified to allow the formation of EE including clathrin-mediated, caveolae-mediated and clathrin-independent endocytosis which are detailed and reviewed in (Elkin et al., 2016; Kaksonen & Roux, 2018). Rab5, a Rab GTPase, associates with the vesicles and promote their fusion with EE (Kohler & Roy, 2015). It recruits and activates Vps34 (vacuolar protein sorting 34), therefore a Rab5 effector, which is a class III PI3-kinase responsible for the production of phosphatidylinositol 3-phosphate (PI3P), on endosomes , that recruits other protein effectors to the vesicle 's membrane (Odorizzi et al., 2000; Shin et al., 2005; Tremel et al., 2021). EEs are also decorated with the early endosome antigen 1 marker (EEA1) (Mu et al., 1995) which is another Rab5 effector that binds to PI3P and is required for proper sorting and fusion of endosomes (Christoforidis et al., 1999). As they mature, they become more acidic through the activity of the v-type vacuolar H^+ ATPase (vATPase) that pumps hydrogen into the vesicle (Fuchs et al., 1989; Mellman et al., 1986). At this point, three sorting options are possible. First, the EE takes the retrograde pathway to go

back to the TGN and possibly after to the ER using COP-I coated vesicles (Aoe, 1997; Gimeno-Agud et al., 2025) (**Figure 17**).

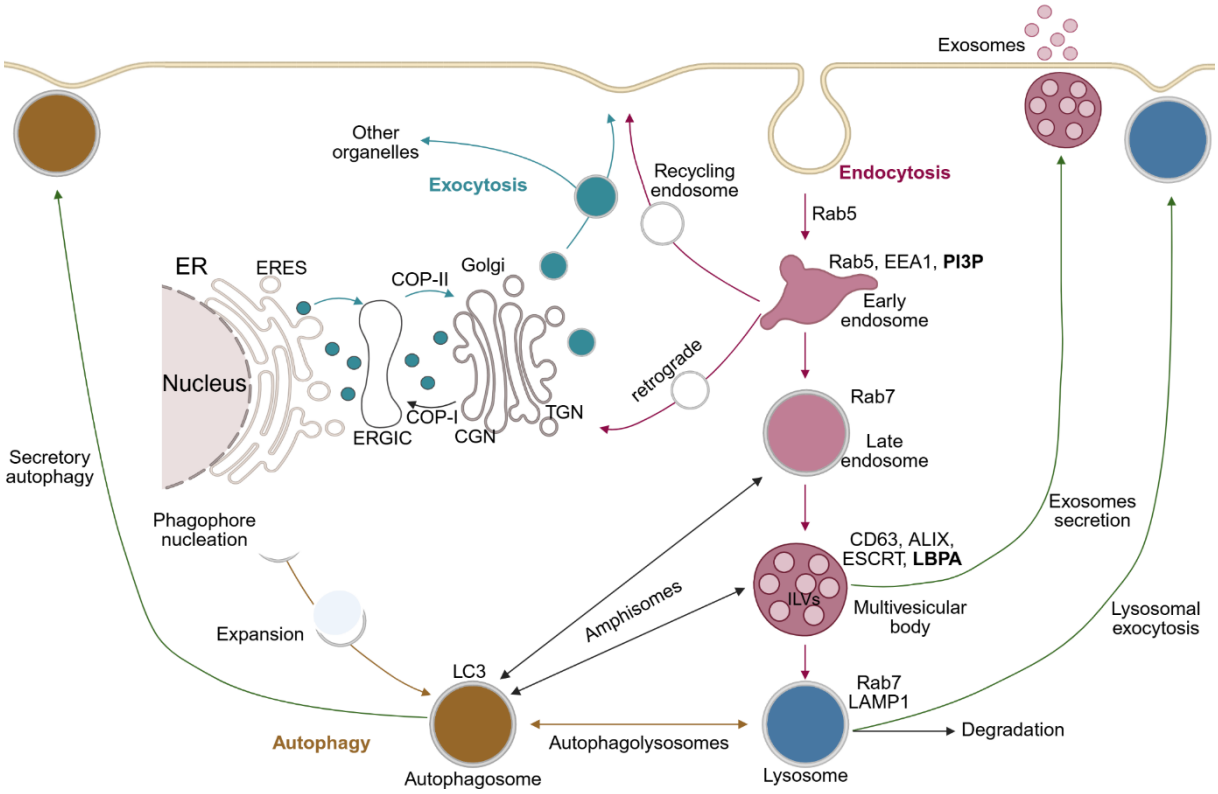

Figure 17. The membrane trafficking pathways within a eukaryotic cell.
The three main trafficking pathways are exocytosis (blue), endocytosis (pink) and autophagy (dark yellow). The key proteins or markers involved at several steps of the trafficking are mentioned. Double-sided arrow means the fusion of the two corresponding vesicles. The green arrows represent the exocytosis of vesicles that do not originate from the 'classical' ER-Golgi secretory pathway. Lipids are in **bold**. ER: endoplasmic reticulum, ERES: ER exit sites, ERGIC: ER-Golgi intermediate compartment, CGN: cis-Golgi network, TGN: trans-Golgi network, ILVs: intraluminal vesicles. Inspired from (Gimeno-Agud et al., 2025). Made with Biorender.

Alternatively, the EE is recycled to the PM in a vesicle known as recycling endosome (RE) (Mayor et al., 1993; Sheff et al., 1999; Taguchi, 2013) (**Figure 17**). The RE cagoes include containing transporters (glucose transporter, (Bryant et al., 2002), receptors (transferrin receptor, (Van Dam et al., 2002) in addition to transmembrane (E-cadherin, (Lock & Stow, 2005) and signaling (GTPase H-Ras, (Misaki et al., 2010) proteins.

The RE fuses with the PM through RE-mediated exocytosis using a specific SNARE (soluble N-ethylmaleimide sensitive factor receptor) machinery in addition to the octameric exocyst complex (Rizo & Südhof, 2012c; Wu & Guo, 2015).The SNARE complex consists of the v-SNARE proteins (vesicle associated) and t-SNAREs present on the PM. The t-SNAREs are syntaxin 4 (STX4) and the synaptosome-associated protein 23 (SNAP23) which interact with the v-SNARE vesicle-associated membrane protein 3 (VAMP3) in the case of a RE (**Figure 18 A**) (S. K. Rao et al., 2004; Taguchi, 2013). Whereas the exocyst complex consists, briefly, of 8 subunits : Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 (Wu & Guo, 2015).

Exo70 and Sec3 interact with the PI(4,5)P2 at the level of the inner leaflet of the PM (B. He et al., 2007; Liu et al., 2007; X. Zhang et al., 2008) while Sec15 localizes to the vesicle (Guo et al., 1999). The rest of the subunits link Sec15 to Sec3 and Exo70 (Mei et al., 2018). Interestingly, Sec15 interacts with Rab11 (X.-M. Zhang et al., 2004), a GTPase that plays an important role in endosomal recycling and trafficking from the TGN to the PM (Ullrich et al., 1996; Urbé et al., 1993). While Sec5 and Exo84 are themselves controlled by the Ral GTPases RalA and RalB (Gentry et al., 2014).

In the third case, the cargo is selected for degradation and therefore the EE matures into late endosome (LE) and is driven to fuse with lysosomes. Rab7 is associated with both LEs and lysosomes (T. Wang et al., 2011), whereas lysosome associated membrane protein-1 (LAMP1) is specific for lysosomes (Eskelinen, 2006). The LE turns into multivesicular bodies (MVBs) upon the intervention of the endosomal sorting complexes required for transport (ESCRT) machinery composed of four protein complexes, each with its respective actors. ESCRT 0 recognizes monoubiquitinated proteins and is recruited to the endosomal membrane. ESCRT I and II stimulate a local membrane invagination and ESCRT III finishes the scission of the membrane leading to the formation of intraluminal vesicles (ILVs) (Urbanelli et al., 2013; Vietri et al., 2020a, 2020b). In addition, ALIX, an accessory protein associates specifically with lysobiphosphatidic acid (LBPA) controls the membrane invagination and organization (**Figure 17**), therefore having a crucial role in ILVs biogenesis (Matsuo et al., 2004). Several studies revealed an ESCRT-independent ILVs formation mechanism that relies on ceramide lipids in raft microdomains (Colombo et al., 2013; Trajkovic et al., 2008).

MVBs either fuse with the PM and release the ILVs known as exosomes in the extracellular environment or fuse with the lysosomes for cargo degradation (Colombo et al., 2014; Villarroya-Beltri et al., 2016). Several tetraspanins such as CD9, CD63 and CD81 are considered markers of extracellular vesicles (EVs) including exosomes (**Figure 17**) (van Niel et al., 2011; Andreu & Yáñez-Mó, 2014). Another possibility is the lysosomal exocytosis (**Figure 18 B**), triggered by elevated intracellular calcium concentration, where lysosomes fuse with the PM using a SNARE machinery similar but not the same as the one used by the RE (VAMP7 instead of VAMP3) (S. K. Rao et al., 2004). When intracellular Ca^{2+} level increases, calcium binds synaptogamin VII that is localized at the vesicles' membrane, inducing a conformational change. This leads to an interaction between the PM, the preformed SNARE complexes and synaptogamin leading to lysosome's fusion (Andrews, 2005; S. K. Rao et al., 2004).

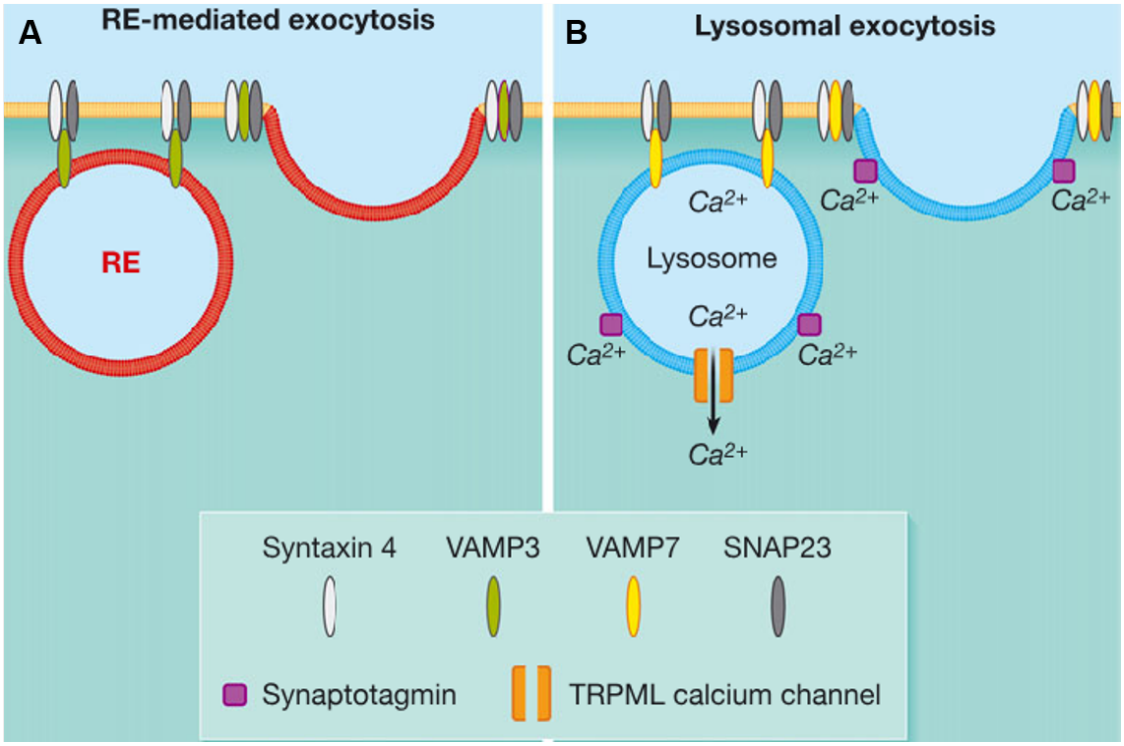

Figure 18. The SNARE machinery involved in RE-mediated exocytosis and lysosomal exocytosis. **(A)**. RE-mediated exocytosis uses the SNARE proteins Syntaxin 4, VAMP3 and SNAP23 to fuse with the PM. **(B)**. The exocytosis of lysosomal content requires the increase of intracellular calcium that will bind to Synaptogamin VII present on the vesicles' membrane leading to the interaction with VAMP7, SNAP23 and Syntaxin 4 of the SNARE machinery. From (Ireton et al., 2018).

3.1.3 Autophagy

Autophagy**,** first defined as a self-eating process by Christian De Duve in 1963 (De Duve, 1963), is a degradative pathway that relies on membrane trafficking and vesicular transport. It enables the degradation and recycling of cellular components such as misfolded proteins and damaged organelles (Wei et al., 2018). Macroautophagy is the most studied autophagy process compared to the other two that are termed microautophagy and chaperone-mediated autophagy (CMA) which will not be described here (Boya et al., 2013). Macroautophagy, herein autophagy, begins with the signaling of a component for degradation which leads to the nucleation of a double membrane that keeps on extending in an open conformation known as the phagophore. Later on, the membrane closes engulfing the cytoplasmic material inside, the famous stage of autophagosome (Feng et al., 2014; Nakatogawa, 2020). Autophagosomes then fuse with lysosomes to form autophagolysosomes where the cargo is degraded and the products are recycled to cells' consumption (Deretic et al., 2013; Yoshimori & Noda, 2008). It is to note that autophagosomes could also fuse with the PM through the « secretory autophagy » process or fuse with MVBs or LE forming amphisomes therefore either they will be released in the extracellular environment or fused later on with lysosomes for degradation (**Figure 17**) (Buratta et al., 2020; J. Xu et al., 2018).

A complex machinery has been identified to shape and control the autophagy process (Amaya et al., 2015; Wei et al., 2018) and some key components are important to define. It is regulated by a family of genes named autophagy-related genes (ATG) (Klionsky et al., 2003). Among them, genes encode for proteins that are the core for this machinery and are divided into 4 sub-groups : 1/ the ATG1/ULK1 complex with ATG13, FIP200 and ATG101, 2/ a class III PI3K complex including Vps34, Vps15, ATG6/Beclin1 and ATG14 leading to the generation of PI3P, 3/ ubiquitin-like conjugation systems leading to the formation of ATG5-ATG12 complex which associates with ATG16L1. This complex then permits LC3 to bind phosphatidylethanolamine (PE) at the autophagosome membrane, with the help of ATG7, ATG10, ATG3 and ATG4 and 4/ a transmembrane protein ATG9. Groups one and two modulate the first step of autophagy; the initiation and biogenesis of the autophagosome, group three plays a role in membrane elongation and autophagosome growth and the last group is involved in membrane extension and autophagosome formation and maturation (Itakura & Mizushima, 2010; Mancias & Kimmelman, 2016; Q. Xiong et al., 2018). Altogether, eleven Rab proteins are involved in autophagosome biogenesis and maturation including Rab5 and Rab7 (reviewed in (Amaya et al., 2015). In addition, several SNAREs play a role in the different steps in autophagosome formation among them SNAP23, VAMP7, STX7 and STX17 (Amaya et al., 2015) (**Figure 19**). Upon autophagy induction, cytosolic LC3 (LC3-I) is conjugated to PE and becomes LC3-II associated to the autophagosome membrane, thus it is a well-known marker of autophagy (Tanida et al., 2008). Another protein of this machinery, p62 (known as sequestosome 1, SQSTM1) is an adaptor protein that recognizes ubiquitinated and targets tagged proteins to the autophagosome (Deretic, 2010; Levine et al., 2011).

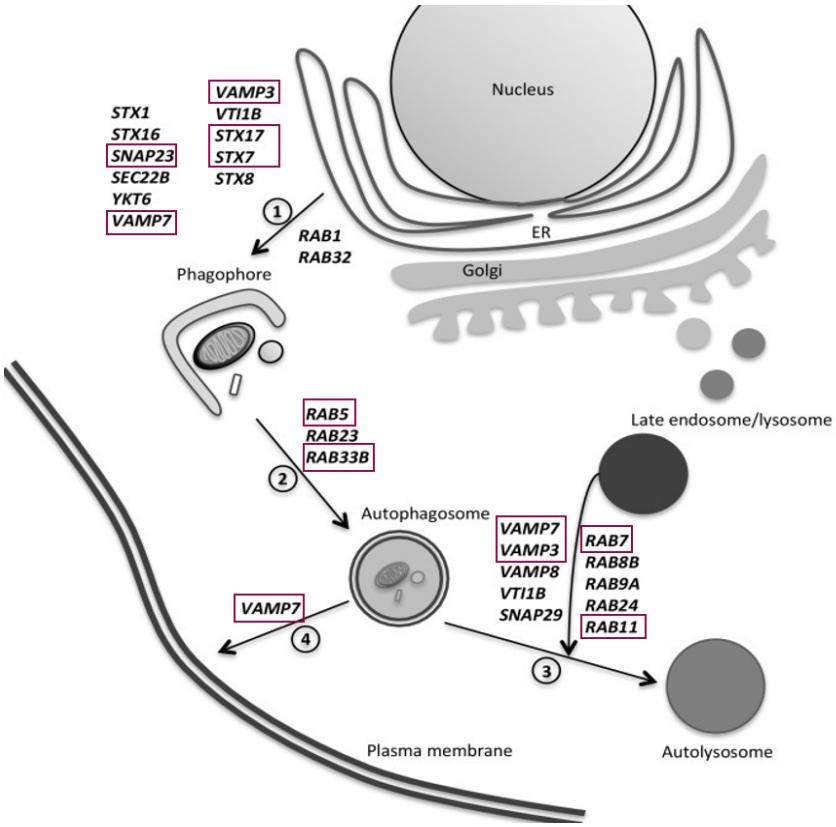

Figure 19. The SNARE and Rab proteins involved in the autophagy process. Scheme highlighting the SNARE and Rab proteins that are implicated at the different steps of autophagosome formation leading to the degradative compartment auto(phago)lysosome. The SNARE and Rab proteins that will be mentioned in the upcoming sections are indicated in red rectangles. Phagophore formation (1), elongation and sealing to generate the autophagosome (2). (3) Autophagosome maturation and possible fusion with the PM (4). From (Amaya et al., 2015).

3.2 Bacteria interactions with the trafficking pathways

The above presented trafficking pathways are often hijacked and/or exploited by bacterial toxins to promote bacteria uptake, replication and survival during infection in addition to host colonization. In parallel, these pathways can act as host defense mechanisms against pathogens.

In the following section, I chose to present one obligate intracellular pathogen (*Coxiella burnetti)*, two facultative intracellular (*Salmonella enterica* serovar Typhimurium and Uropathogenic *Escherichia coli*) and one extracellular (*Pseudomonas aeruginosa*) to highlight the diverse host trafficking machineries implicated in such host-pathogen interactions. Other criteria included that each of the pathogens engages with the endocytic and/or exocytic pathways in addition to the autophagy machinery, shedding light on how one bacteria can be simultaneously involved with multiple pathways. Lastly, some of the host proteins introduced here will be relevant for the results obtained during my thesis. I will be covering a part of what is known about the interaction of these pathogens with the host trafficking machinery. It is to note that there are other intracellular and extracellular pathogens harboring a variety of virulence factors and toxins and are studied in this context. These pathogens will not be presented here and include *Shigella flexneri, Yersinia species, Mycobacterium tuberculosis,*

Bacillus anthracis, Legionella pneumophilia, Staphylococcus aureus, Vibrio cholera and Brucella abortus.

3.2.1 *Coxiella burnetti*

Coxiella burnetti is an interesting example of intracellular pathogen manipulating host trafficking pathways. *Coxiella* is less known that the famous *Legionella pneumophilia* that has been extensively studied in this context (reviewed in (Bärlocher et al., 2017; Iyer & Das, 2021; Weber & Faris, 2018) and it delivers a broad repertoire of effectors.

Coxiella is a Gram-negative obligate intracellular infectious bacterium responsible for a life-threatening flu-like disease known as Q fever (van Schaik et al., 2013). *Coxiella* is internalized by host cells into membranous compartments that follow the endocytic degradative pathway (Howe et al., 2010; P. S. Romano et al., 2007; Schulze-Luehrmann et al., 2016). *Coxiella* uses a sophisticated nanomachinery called Type 4B Secretion System (T4BSS) or Icm/Dot (intracellular multiplication / defect in organelle trafficking genes) to translocate more than 140 effector proteins into the host cytoplasm (Larson et al., 2016), to generate the *Coxiella*-containing vacuoles (CCVs). The membranous compartments expand and *Coxiella* replicates within the CCV with autolysosome-phagolysosome traits in acidic conditions (Berón et al., 2002; Howe et al., 2010; Schulze-Luehrmann et al., 2016). Therefore, many studies investigated the CCVs, their lipid composition and the roles of T4BSS effectors. For instance, CvpB (also known as Cig2) is an effector that recruits PI3P of early endosomes on the CCV by affecting the PIKfyve kinase responsible for the conversion of PI3P into PI(3,5)P2 (Martinez et al., 2016) leading to CCVs expansion. Also, CCVs have several specific markers on their surface : vATPase, LAMP1, CD63 and LC3 in addition to Rab5 and Rab7 (**Figure 20**) which modulate the fusion of membranes with early and late endosomes respectively, as the CCV matures *via* the endocytic pathway (Berón et al., 2002; Howe et al., 2010; Kohler & Roy, 2015).

In a recent study, phosphatidylinositol-4-phosphate (PI4P) and lysobisphosphatidic acid (LBPA) lipids have been demonstrated to be brought to the CCV's membranes in a T4BSS-dependent manner. A new T4BSS effector, termed « Vice » for Vacuole-inducing *Coxiella* effector, has been identified to recruit LBPA to CCVs in addition to the expansion and stabilization of CCVs during infection (Bienvenu et al., 2024). Specifically, Vice induces the formation of VICs (Vice-induced compartments) very similar to CCVs in terms of composition and morphology. Vice recruits and interacts with PS, LBPA and PIPs: PI3P, PI4P and PI5P, lipids shown to be present on VICs. Lastly, Vice enhances macropinocytosis, a non-selective endocytic process of engulfment of extracellular medium (Kay, 2021), probably to deliver membranes for CCV expansion through its C-terminal domain (Bienvenu et al., 2024).

Furthermore, VICs membranes contain Rab7 and LBPA (Bienvenu et al., 2024), two markers of MVBs biogenesis (Matsuo et al., 2004). Further analysis showed that CD63 and TSG101, an ESCRT-I component, localized at the contour of CCVs whereas ALIX was mainly found present in the cytoplasm (**Figure 20**). In addition, Vice directly interacts with ALIX and CHMP3 (an ESCRT-III component). Therefore, the second function of Vice is to stabilize the CCVs by accumulating the ESCRT machinery at the CCVs therefore inhibiting its activity and delaying ILVs formation through its N-terminal domain (Bienvenu et al., 2024).

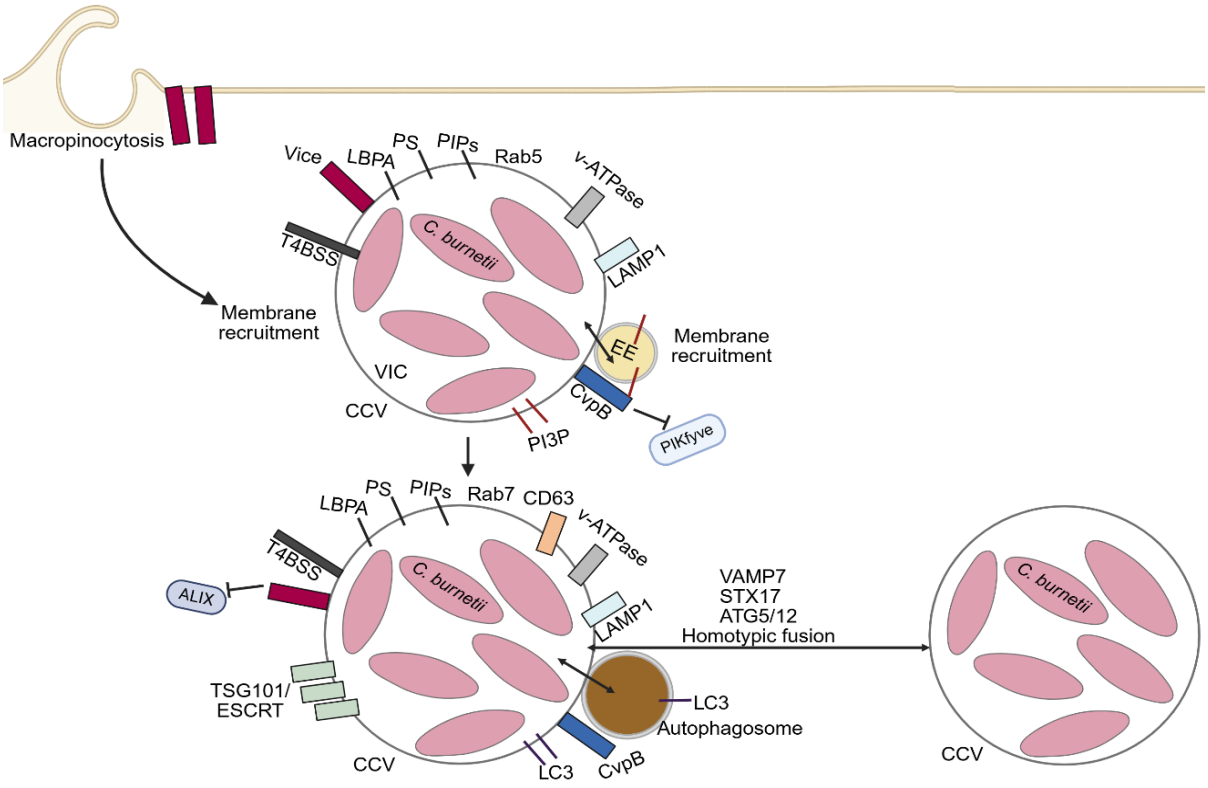

Figure 20. The role of the T4BSS effectors of *Coxiella Brunetii* in manipulating host trafficking pathways.
Coxiella burnetti replicates within *Coxiella*-containg vacuoles (CCVs) in acidic conditions and specific protein and lipid markers. The scheme highlights the dual role of each *Coxiella* T4BSS effector Vice (in red) and CvpB (in blue) during host colonization. Both recruit membranes for vacuolar expansion: Vice through macropinocytosis and CvpB through PI3P interaction and recruitment of PI3P membranes such as EE. Vice blocks ILVs formation (ALIX and ESCRT machinery) to stabilize the CCV. CvpB favors the fusion with LC3 decorated autophagosomes in addition to homotypic fusion with other CCVs. Double sided arrow indicates the fusion of the respective vesicles. Compartments where Vice is present are termed VICs for Vice-induced compartments. Made with Biorender.

Coxiella has also been studied in the context of interaction with the autophagic machinery. First, CCVs membrane is decorated with the LC3 autophagosome marker (P. S. Romano et al., 2007) which is in accordance with the idea that autophagosomes fuse with CCVs in a CvpB-dependent manner (Newton et al., 2014). These fusions lock the expanding CCVs in an autolysosomal phase and are not required for *Coxiella* replication *per se* (Kohler et al., 2016; Newton et al., 2014). In addition to fusion with autophagosomes, CCVs fuse with each other, a process known as homotypic fusion to form a large single compartment that occupies the

majority of host cytoplasm (Howe et al., 2003). Homotypic fusion involves the CvpB T4BSS effector (Newton et al., 2014) in addition to two host cell SNAREs' VAMP-7 and Syntaxin-17 (Campoy et al., 2013; Newton et al., 2014) and two autophagy related genes Atg5 and Atg12 (Newton et al., 2014) therefore higlighting a dual role for CvpB. These host factors showcase the importance of autophagy in the formation of mature CCVs, again by recruiting LC3 and keeping *Coxiella* in an autolysosomal stage, allowing it to be highly fusogenic and therefore promoting its homotypic fusion (**Figure 20**).

The roles of many *Coxiella* T4BSS effectors remain unknown especially because of functional redundancy thus further research is required to explain the complexity of this pathogen. In addition, I believe it is crucial to get insights into how these different interactions are chronologically regulated and balanced to provide the suitable conditions for CCVs and conserve the host as long as it is needed.

3.2.2 Uropathogenic *Escherichia coli* (UPEC)

Unlike *Coxiella*, UPEC is a model of facultative intracellular pathogen equipped with a variety of virulence factors including secreted proteins in addition to extracellular appendages pili, fimbriae and flagella (Mitiku & Lata, 2025). It is a common cause of urinary tract infections (UTIs) that uses its virulence factors to colonize the bladder and escape host's immune responses (Whelan et al., 2023). UPEC takes advantage of the bladder volume regulation that occurs when the bladder is filled with urine, as the epithelium must extend. Rab27b intracellular vesicles known as fusifom vesicles fuse with the membrane of epithelial cells to provide additional membrane for extension. When the bladder is emptied, the membranes are interanlized again englufing with them adhered UPEC (Bishop et al., 2007; Y. Chen et al., 2003). UPEC's internalization is supported by the virulence factors: the toxin cytotoxic necrotizing factor 1(CNF1) and the adhesisns Afa/Dr and the type 1 pili adhesin FimH. CNF1 continuously activates Rho GTPase leading to augmented cell spreading and cytoskeletal rearrangments enhancing UPEC's internalization (Doye et al., 2002; Schmidt et al., 1997). As for the adhesins, Afa/Dr mediate the attachment of UPEC to the host *via* the recognition of host α5β1 integrin and/or CD55 (Guignot et al., 2001; Kansau et al., 2004) and FimH through CD48 receptor in macrophages (Malaviya et al., 1999).

Upon internalization, UPEC resides in vacuoles decorated with Rab27b GTPase (J. Song et al., 2009) which modulates host protein secretion specifically by regulating the vesicles' transport and docking steps (Underwood et al., 2020). On the same vesicles, the Toll-like receptor 4 (TLR4) is detected (J. Song et al., 2009) which is a pattern recognition receptor (PRR) that recognizes pathogen associated molecular patterns (PAMPs) (J. Zhu & Mohan, 2010). In the case of UPEC, it senses the lipopolysaccharide (LPS) present on its envelope

(Whelan et al., 2023). One way the host reacts to UPEC is through TLR4-mediated expulsion (Miao et al., 2016).

TLR4 usually initiates a type I interferon response through adaptor proteins TRIF and TRAM in addition to TRAF3 which is an E3 ubiquitine ligase (Kawai & Akira, 2011). As the downstream effectors of TRAF3 are not present on the vacuole, type I interefron response is impossible. This leads to a K33-linked (lysine 33) ubiquitination of TRAF3 and therefore the formation of a complex between TRAF3 and RalGDS (guanine exchange factor for Ral GTPases) (Gentry et al., 2014; Miao et al., 2016). The ubiquitination of TRAF3 activates and recruits RalB GTPase (Miao et al., 2016) which is a positive regulator of the exocyst complex (Wu & Guo, 2015). The two mentioned RalB and RalGDS in addition to Sec5, Sec84 and Sec8 of the exocyst were shown to have a valuable role in this process of expulsion. Rab11a and Rab27b are recruited and activated by Sec6 and Sec15 (**Figure 21**). Rab27b interacts with MyRIP which associates with the motor protein Myosin VIIA (microtubule) (Welz et al., 2014). Rab11a interacts with Rab11FIP3 which associates with the motor protein dynein (actin filament) (Welz et al., 2014). This eventually leads to the expulsion, from host cell, of UPEC through the recruited exocyst complex (Miao et al., 2016), which is an example of trafficking-dependent efficient host defense mechanism.

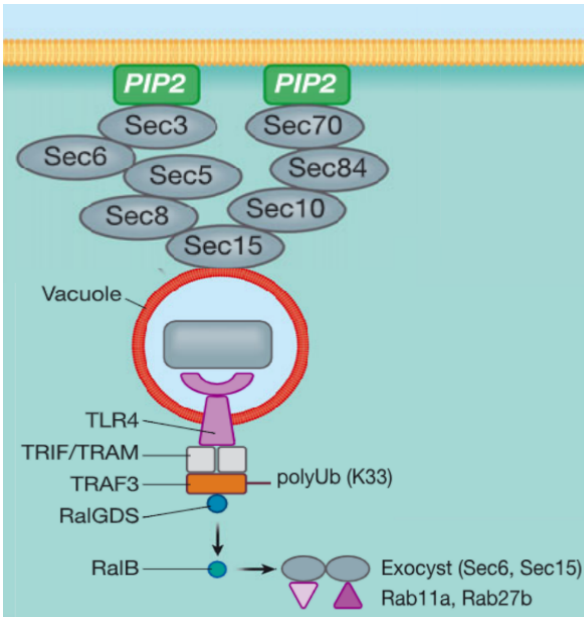

Figure 21. The exocyst complex- and TLR4-mediated expulsion of UPEC.
The sensing of UPEC by the TLR4 leads to the ubiquitinyation of the Lys-33 (K-33) of TRAF3 therefore activates the exocyst positive regulator RalB GTPase. Exocyst proteins Sec6 and Sec15 recruit and activate Rab11a and Rab27b GTPases that mediate the exocytosis of the vacuole. From (Ireton et al., 2018).

UPEC could escape from the Rab27b vesicles, avoid the first expulsion process, progress in the cell infection and localize at 2 hours post infection with autophagic compartments (Miao et al., 2015). At 4 hours post infection, UPEC is found in CD63 and LC3-decorated compartments and is subsequently found in LAMP1-labelled vacuoles. These vacuoles have a neutral pH which activates a specific lysosomal calcium channel TRPML3, a channel that increases intracellular calcium which is sensed by synaptogamin VII. Therefore, bacteria are expelled in

a process like the lysosomal exocytosis (described in 3.1.2), this one distinguished by the release of lysosomal enzymes and the increase of LAMP1 at the PM. The expulsed UPEC associate with host membrane structures that are positive for CD63 and ESCRT components markers, making them similar to exosomes (Miao et al., 2015). This suggests that bacteria are encased in autophagosomes then into MVBs and lastly in lysosomes pinpointing an unusual trafficking route for UPEC elimination. Recently, an UPEC phospholipase PldA was shown to facilitate both the TLR4-mediated exocytosis (Pang et al., 2022) and the lysosomal exocytosis escape of UPEC (X. Li et al., 2024).

In addition, studies demonstrated that the autophagy gene *ATG16L1* is required for the survival of UPEC in infected mice bladders suggesting a role of the autophagy machinery. The lack of ATG16L1 is accompanied by an increased stimulation of the innate immune response and a strong proinflammatory response dependent on IL-1β cytokine leading to a better elimination of UPEC (Symington et al., 2015; C. Wang et al., 2012). In addition, UPEC's persistence requires ATG16L1 and ATG7. A mutation in the ATG16L1 lead to an increased expression of secretory Rab GTPases involved the expulsion of UPEC previously described in addition to Rab33B that plays a role in autophagosome formation leading therefore to a successful clearance of UPEC (C. Wang et al., 2019) (**Figure 22**). This suggests that UPEC overcomes these cellular responses in basal levels of expression and that its hijacks the autophagic machinery. Furthermore, UPEC enters along with iron-bound ferritin into lysosomes and autophagosomes. Iron is released from ferritin through ferritinophagy and becomes available for UPEC which stimulates its excessive proliferation and eventually eukaryotic cell death (Bauckman & Mysorekar, 2016). This is probably ensured by the TonB-dependent iron acquisition system (Torres et al., 2001). Lastly, it was proposed that UPEC subverts the recycling endosome pathway (described in 3.1.2) of transferrin receptor mediated by Rab35 to acquire iron and avoid lysosomal fusion (Dikshit et al., 2015) (**Figure 22**).

Interestingly, not many UPEC proteins/factors are reported to be involved in these interactions with the host trafficking pathways. In addition, the link between UPEC and autophagy is not well established yet. Therefore, future studies can focus on the bacterial pathogen to better understand the mechanisms governing its dynamics with the host.

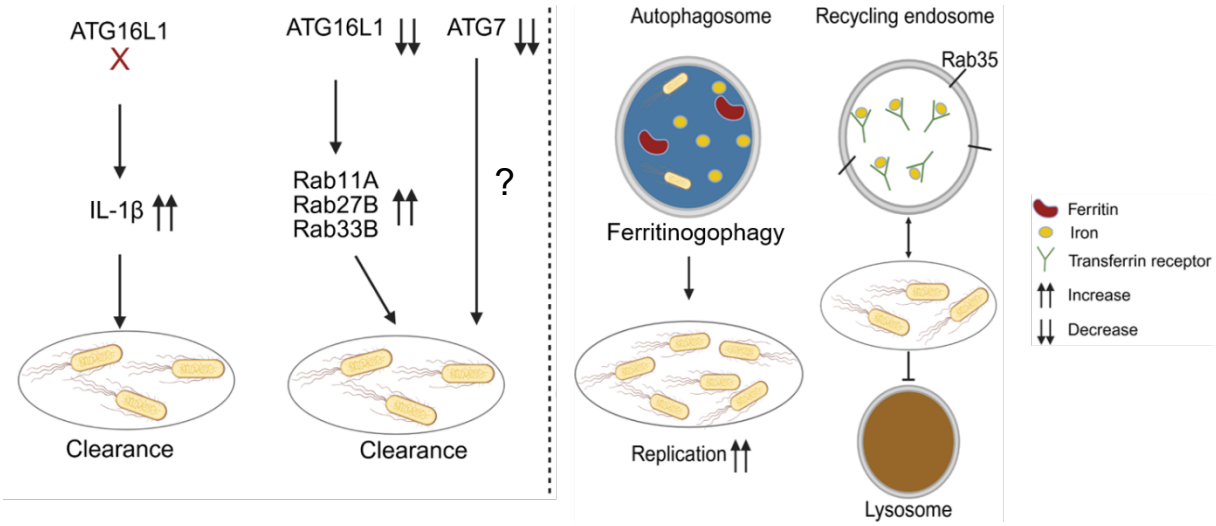

Figure 22. The relationship between the host's autophagy machinery and UPEC.
Summary of what has been observed so far concerning the dynamics between autophagy and UPEC during host colonization. ATG16L1 and ATG7 levels affect the clearance of UPEC from host cells. UPEC takes advantage of host ferritinophagy and recycling endosome to replicate and avoid lysosomal fusion respectively. Double-sided arrow means the fusion of the two concerned vesicles. Made with Biorender.

3.2.3 *Salmonella enterica* serovar Typhimurium

Another facultative intracellular pathogen known for its manipulation of host trafficking pathways is *Salmonella enterica* serovar Typhimurium (herein *Salmonella*). It is responsible of most food-borne illnesses in the whole world and causes strong diarrhea and enteric fever (Fàbrega & Vila, 2013). *Salmonella* possesses a vast repertoire of virulence factors including two T3SSs encoded within two separate *Salmonella* pathogenicity islands (SPI-1 and SPI-2) each with its respective effectors (Marcus et al., 2000) and a total of 49 effectors (reviewed in (Worley, 2025). SPI-1 is involved at the initial invasion phase whereas SPI-2 manipulates host trafficking pathways for the sake of *Salmonella*'s survival and replication (Marcus et al., 2000). Once within the host, *Salmonella* thrives within membranous structure termed *Salmonella*-containing vacuole (SCV) decorated with LAMP1. A subpopulation can escape the SCV and replicate in the cytoplasm (Beuzón et al., 2002; Brumell et al., 2002; Castanheira & García-Del Portillo, 2017).

The SPI-1 SopB effector, during invasion, recruits Rab5 and Vps34 to the SCV leading to PI3P in the SCV membrane in addition to the LAMP1 marker (Bakowski et al., 2010; Mallo et al., 2008). The SPI-2 effector SpvB affects the endocytic pathway by inhibiting clathrin-mediated endocytosis and the secretory pathway by impacting the anterograde trafficking from the TGN to the PM (Yuan et al., 2023). SopD2, a SPI-2 effector, acts as an inhibitor of two GTPases. It blocks the Rab7-mediated SCV fusion with LE or lysosomes by hindering its interaction with its effectors RILP and FYCO1, dynein and kinesin-binding proteins (D'Costa et al., 2015). It cooperates with GtgE protease to block Rab32 accumulation on the SCV most likely to hamper the delivery of anti-bacterial molecules to the vacuole (Spanò et al., 2016). Another "famous"

SPI-2 effector, SifA is essential for the formation of SIFs (*Salmonella*-induced filaments) and the SCV's integrity (Beuzón et al., 2002). SIFs are tubule-like structures protruding from the SCV (Garcia-del Portillo et al., 1993). SifA directly binds SKIP (SifA and kinesin–interacting protein) to manipulate kinesin motor activity favoring tubules biogenesis (SIFs) (Boucrot et al., 2005). The SifA–SKIP interaction confiscates Rab9 at the level of the SCV disturbing the retrograde trafficking, including the delivery of hydrolases and proteases to lysosomes (McGourty et al., 2012) (**Figure 23**). Both SifA and SopD2 interact with STX7 (D'Costa et al., 2019). Infecting cells devoid of STX7 lead to a reduced invasion and replication of *Salmonella*. This suggests that *Salmonella* exploits STX7 presumably in order to initiate an intracellular niche, steer clear of lysosomes and acquire crucial nutrient sources for its intracellular replication (Vij et al., 2025).

Several *Salmonella* effectors are known to interact with autophagic machinery of the host and interfere with the autophagy at different steps (Herhaus & Dikic, 2018; Sorbara et al., 2018; Yu et al., 2014) . A study by Yu et al, showed that in the absence of autophagy factors ATG5, LC3, ATG16L1 or p62, cytosolic *Salmonella* replicative ability is reduced. Cytosolic *Salmonella* interacts specifically with LC3 and p62 from the autophagy machinery to replicate (Yu et al., 2014) and SopB is essential for this association (Yu et al., 2014). Yet, *Salmonella* (SCVs) infected cells present a SopB-mediated decreased autophagy and are protected from lysosomal fusion (Chatterjee et al., 2023). SopB also decreases the amounts of PI(4,5)P2 and PS present on the SCV. This modification of the charge of the SCV's surface inhibits the Rab GTPases (Rab 8B, 13, 23 and 35) leading to SCV's lysosomal escape (**Figure 23)** (Bakowski et al., 2010; A. C. Smith et al., 2007). Lastly, it was proposed that autophagy plays a role in the repair of SCV membranes, that are damaged by the T3SS of SPI-I passing though it (Kreibich et al., 2015).

A recent study highlighted an unnoticed role of the outer membrane protein A (OmpA) of *Salmonella*. OmpA stabilizes the SCV through an important interaction with LAMP1 and protects it from lysosomal clearance. Also, OmpA allows cytosolic *Salmonella* to escape from lysosomal fusion (Roy Chowdhury et al., 2025).

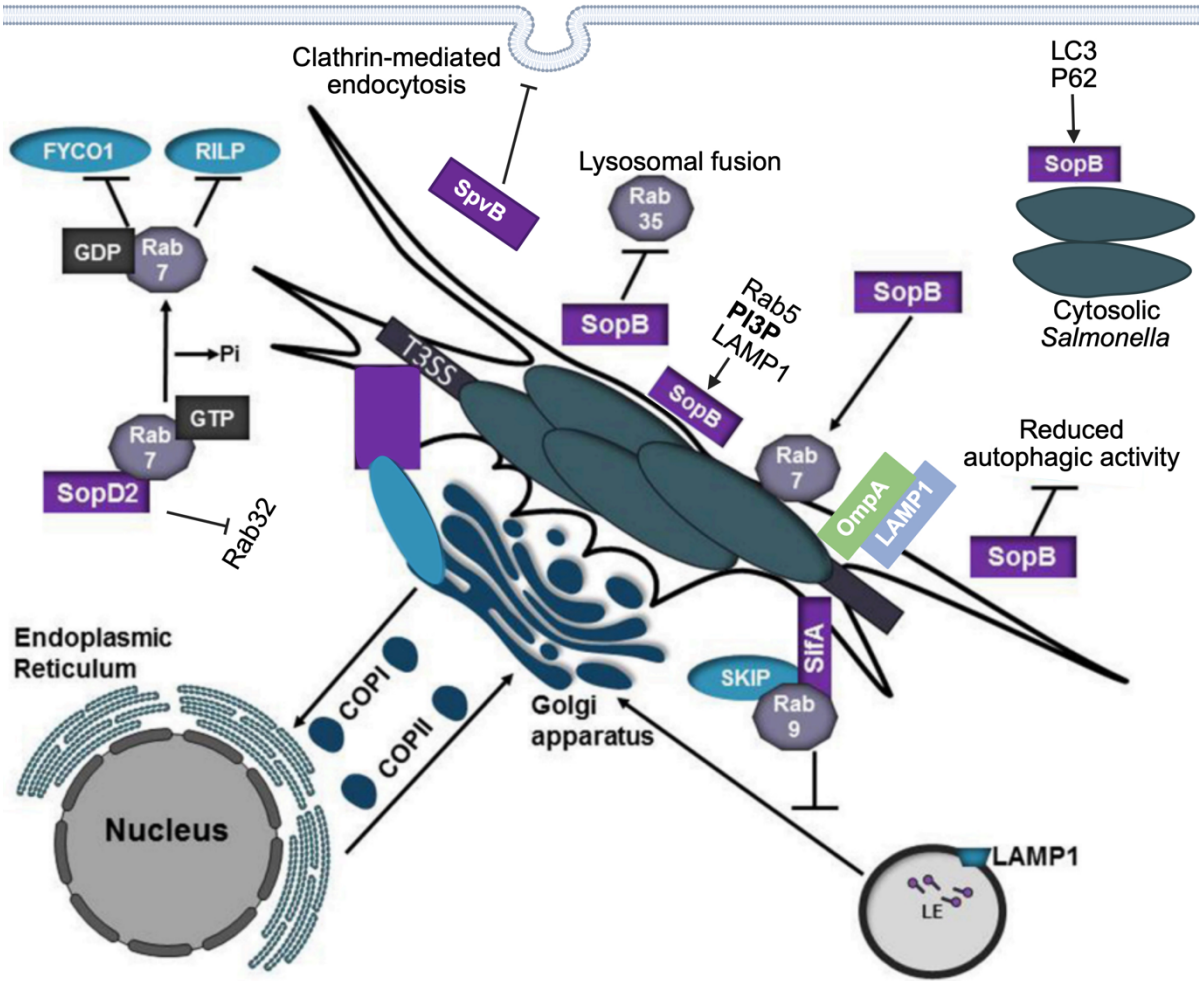

Figure 23. The T3SS effectors of *Salmonella* manipulate host trafficking pathways and favor its survival.
SopB recruits proteins/markers and PI3P to the *Salmonella*-containing vacuole (SCV), it changes the charge of the SCV's surface and therefore protects it against lysosomal fusion by hindering the targeting of Rab35. SopB is also required for cytosolic *Salmonella* association with host autophagy machinery but reduces the autophagic activity of the host protecting the SCVs. SpvB interferes with the endocytic pathway by inhibiting the clathrin-mediated endocytosis. SopD2 inactivates Rab7 by blocking its interaction with its effectors and blocks Rab32 accumulation on the SCV. SifA interacts with SKIP and sequester Rab9 thus inhibiting retrograde trafficking. OmpA stabilizes and protects the SCV by interacting with LAMP1. Adapted from (Weber & Faris, 2018).

3.2.4 *Pseudomonas aeruginosa*

P. aeruginosa uses the T3SS to inject toxins into host cells but it is a model for extracellular opportunistic pathogens. Two of *P. aeruginosa*'s T3SS effectors have been studied in more details for their intracellular trafficking and activities - ExoS and ExoU.

3.2.4.1 ExoS toxin

The trafficking of the ExoS toxin of the T3SS of *P. aeruginosa* (previously described in section 2.3.1) was investigated throughout the years. Interestingly, ExoS was first observed to localize with the alpha-mannosidase Golgi enzyme and to the perinuclear region (Pederson et al.,

2002). ExoS also localizes with endosomal markers, Rab9, Rab5 and Rab6 which pointed towards a retrograde trafficking to the perinuclear region (Y. Zhang et al., 2007) and urged deeper understanding of ExoS trafficking.

The proposed model for ExoS trafficking within the eukaryotic host claims that ExoS accumulates at the PM in a transient manner. Then, ExoS travels to the perinuclear region, in retrograde, using host microtubules network and avoiding any acidification process on Rab decorated endosomes. Microtubules allow the maintenance of ExoS in close proximity to the perinuclear region where it ADP-ribosylates Ras. This allows ExoS to reach the ER without passing by the Golgi apparatus while interacting with its targets at different time points. ExoS could recycle back from the Golgi/ER to the PM, in a yet unknown mechanism, probably to reach its plasma membrane localized targets such as Ras (**Figure 24**) (Q. Deng et al., 2007) and probably the ERM proteins (ezrim, radixin and moesin) close to the PM.

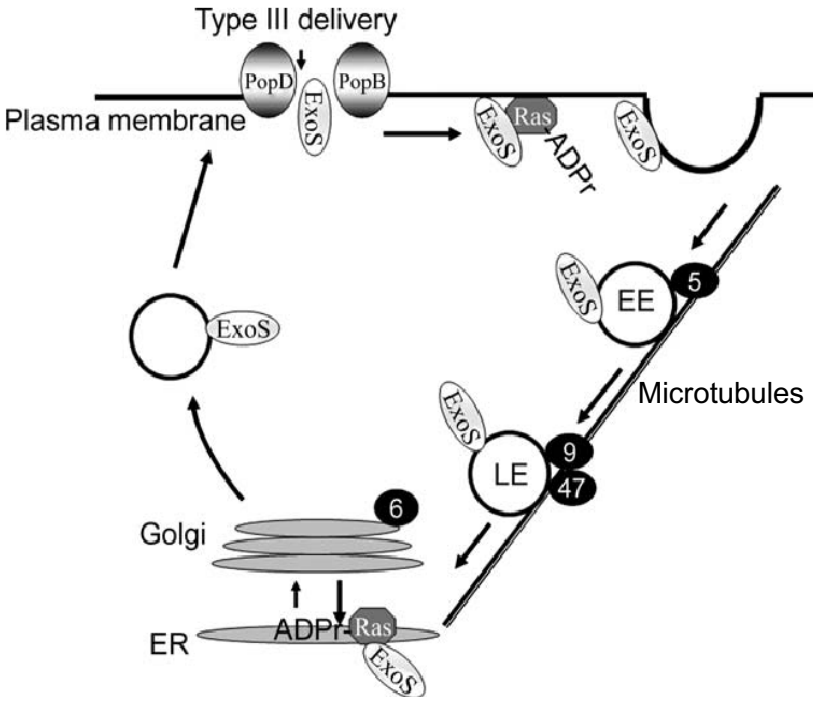

Figure 24. The travel of ExoS within the eukaryotic host.
The proposed model suggests that upon injection, ExoS localizes to the PM where it can interact with its targets. It traffics back to the perinuclear region using microtubules and endosomes decorated with different Rabs (Rab5, 6 and 9) and can reach the ER where it ADP-ribosylates Ras. ExoS traffics back to the PM in an unknown mechanism. EE: early endosome, LE: late endosome. From (Q. Deng et al., 2007).

ExoS ADP-ribosyl transferase activity was shown to inhibit the activity of the Rab GTPase, Rab5 protein. This way, the newly internalized material through endocytosis (clathrin –coated vesicles and fluid-phase uptake) does not mature into early endosome probably by blocking the interaction Rab5-EEA1, therefore leading to the disruption of the endocytic pathway (Q. Deng & Barbieri, 2008). In fact, ExoS ADP-ribosylates Rab5 on 4 arginine residues (81, 91,

110 and 120) and blocks it in GDP-bound inactive form. Yet, active Rab5 is not required for the trafficking of ExoS within the host (Simon & Barbieri, 2014).

In addition to endocytosis manipulation, the effect of ExoS on autophagy was investigated. Impressively, the ADPRT domain of ExoS is enough to attenuate host autophagy process and protect *P. aeruginosa* from elimination. More precisely, ExoS ADPRT domain clearly reduced the LC3-II autophagosomal marker generation (L. Rao et al., 2021). The ADPRT activity of ExoS inhibits the kinase activity of the class III phophatidylinositol 3-kinase Vps34 in the Vps34-Beclin1-Atg14L complex that plays a role in autophagosome initiation (**Figure 25**) (L. Rao et al., 2021). Vps34 produces phosphatidylinositol 3-phosphate (PI3P), a lipid enriched on a specific compartment preceding autophagosome formation where autophagic proteins are recruited (Itakura & Mizushima, 2010; Y.-M. Kim et al., 2015). This showcased the manipulation of the autophagic machinery by the T3SS ExoS toxin of *P. aeruginosa*, an unexpected function of the ADPRT domain of ExoS. Yet the molecular mechanism that is occurring to block the Vps34 kinase activity is not determined.

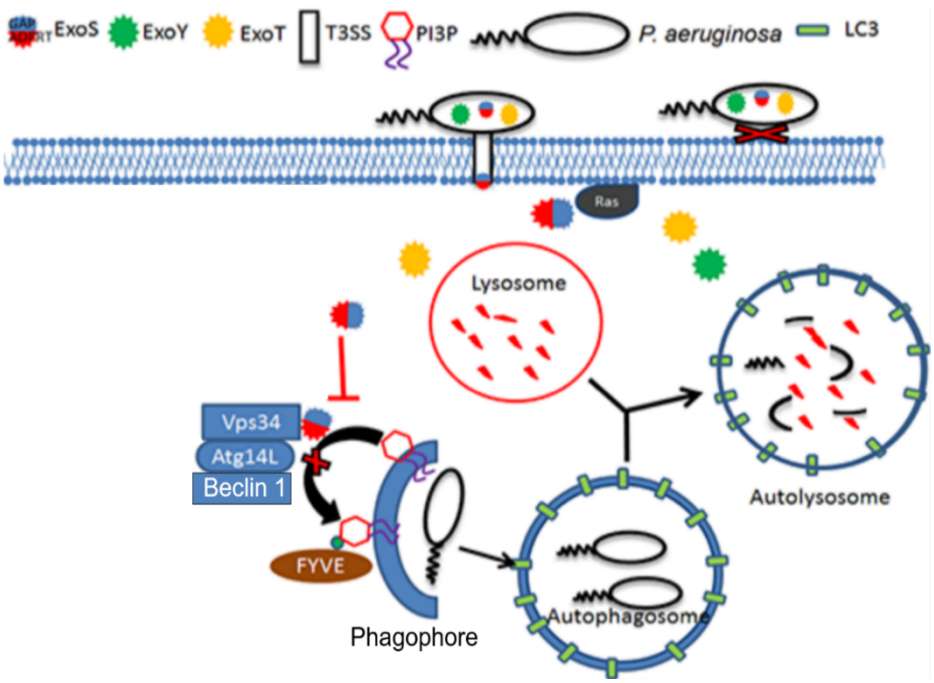

Figure 25. ExoS ADPRT activity inhibits Vps34 kinase of the autophagy machinery.
ExoS ADPRT activity disturbs the Vps34 kinase within the Beclin1-Atg14L-Vps34 complex involved in autophagosome formation. Vps34 produces PI3P that accumulates on the newly formed phagophore membrane. This inhibition leads to a decreased host autophagy and therefore to *P. aeruginosa* survival. Adapted from (L. Rao et al., 2021).

3.2.4.2 ExoU and the Misfolding-associated protein secretion (MAPS) pathway

MAPS is an unconventional protein secretion pathway first defined in 2016 (J.-G. Lee et al., 2016). It consists of the elimination, by secretion, of cytosolic misfolded proteins from cells' cytoplasm to avoid their accumulation (J.-G. Lee et al., 2016). Several MAPS actors have been identified so far and therefore a mechanism of MAPS functioning was proposed by (J.-G. Lee et al., 2016; Ye, 2018). Misfolded proteins are first recruited to the ER surface by the deubiquitinase USP19 and its chaperone HSC70. The proteins are then transferred to the cysteine string protein (CSP also named DNAJC5) which is associated to the membrane of LE through the palmitoylation of the cysteine string motif (**Figure 26 A**). It was proposed that the DNAJC5-cargo complex then enters the lumen of the vesicle, that is directed towards the PM to which it fuses to release its content, including the DNAJC5-cargo complex, to the extracellular space (**Figure 26 B**) (Ye, 2018). Alternatively, the DNAJC5 vesicles can be secreted as EVs (J. Deng et al., 2017). Several studies have shown that Tau and α-synuclein (α-syn) proteins are secreted through the MAPS pathway (Fontaine et al., 2016; J. Lee et al., 2023; J.-G. Lee et al., 2016; Y. Xu et al., 2018). Tau is the protein associated with Alzheimer's disease and α-syn is a misfolded protein associated with Parkinson's disease (Braak & Braak, 1991; Polymeropoulos et al., 1997). DNAJC5 is an essential component of the MAPS pathway, as tau release from neurons was reduced in DNAJC5 KO cells (Fontaine et al., 2016). It contains three domains (**Figure 26 A**) each one with its respective function (Burgoyne & Morgan, 2015); the N-terminal domain with two phosphorylation sites serine 10 and serine 34 that is present within the J domain that interacts with Hsp70/Hsc70 chaperones (Chamberlain & Burgoyne, 1997). It is followed by a central cysteine string that is palmitoylated allowing DNAJC5 to anchor membranes (Greaves et al., 2008). Lastly, the C-terminal domain interacts with SNARE proteins to mediate the vesicles' fusion with the PM (Boal et al., 2004). DNAJC5 mutations are associated with a neurodegenerative disease known as adult neuronal ceroid lipofuscinosis (ANCL) (Benitez et al., 2011; Nosková et al., 2011). In this disease, L115R or ΔL116 mutations in the cysteine string domain of DNAJC5 abrogate the trafficking of DNAJC5 vesicles to the PM and gradually lead to protein accumulation and neuronal dysfunction (Greaves et al., 2012).

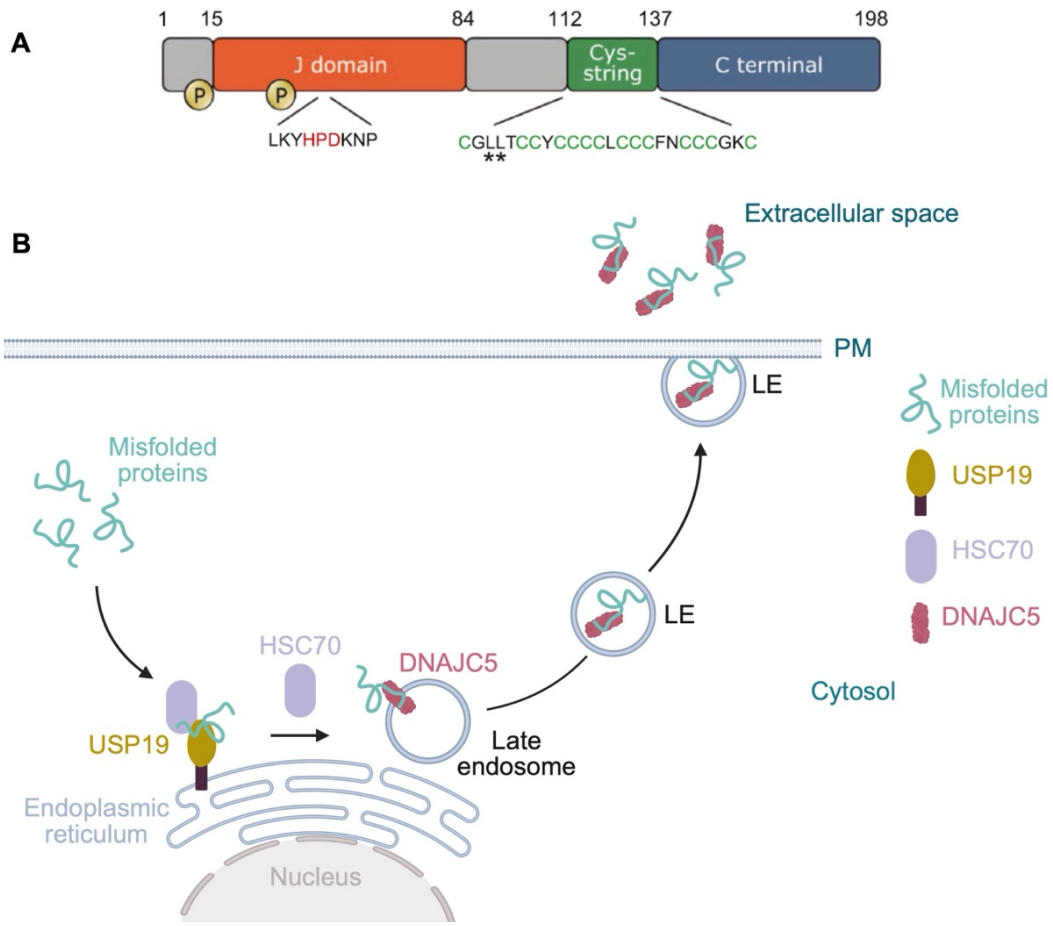

Figure 26. DNAJC5 and the MAPS pathway.

(**A**). Schematic representation of the domains of the DNAJC5 protein. The two phosphorylation sites are marked by 'P'. The HPD residues in the J domain allow the interaction with the chaperones. The stars in the cys-string domain mark the two residues that cause ANCL when mutated (L115 and L116). From (Deruelle et al., 2021). (**B**). The different steps of the MAPS pathway. Misfolded proteins are recruited to the ER (endoplasmic reticulum) and bind USP19 which has a chaperone activity. HSC70 may aid in the USP19-mediated recruitment of misfolded proteins. Misfolded proteins are then transferred with to DNAJC5 which is a co-chaperone of HSC70 and is localized to LE (late endosome). The DNAJC5-cargo complex moves into the lumen of the LE. LEs are then trafficked to the PM where they fuse and release their content in the extracellular milieu. Adapted from (Ye, 2018). Made with Biorender.

As previously described in section 2.3.4, ExoU is a T3SS powerful phospholipase that interacts with its bacterial chaperone in *P. aeruginosa*. It is unfolded and secreted through the T3SS needle. Once injected into the host cell cytoplasm, it is refolded, ubiquitinated and trafficked in an unknown mechanism to the PM where ExoU is activated by the membrane lipid PI(4,5)P2.

Through its travel, ExoU is ubiquitinated at Lys 178 and at its C-terminal domain. It was observed that ExoU ubiquitinated on Lys178 colocalizes with EEA1 marker which pointed towards the involvement of endosomal pathway (Gendrin et al., 2012). More recently, a genome-wide CRISPR-Cas screen in epithelial A549 cells identified DNAJC5 as essential for the delivery of ExoU to the PM through MAPS and in an HSC70-independent manner (Deruelle et al., 2021). ExoU localized with DNAJC5^{+} vesicles at the perinuclear region (also positive for

LAMP2 LE/lysosome marker) and at cellular periphery (Deruelle et al., 2021). This work highlighted the fact that ExoU is hijacking the cellular MAPS pathway allowing it to reach the PM and exert its phospholipase activity (**Figure 27**). Despite this, many questions remain to be answered concerning the ExoU travel within the host. This includes elucidating the mechanisms mediating the interaction with the MAPS vesicles and identifying additional host factors involved in this travel given that known MAPS proteins such as the chaperone HSC70 were not found required for ExoU cytotoxicity. In addition, no direct interaction between DNAJC5 and ExoU could be evidenced using several methods, leaving an open question on additional players in ExoU voyage from the sote of injection to its target.

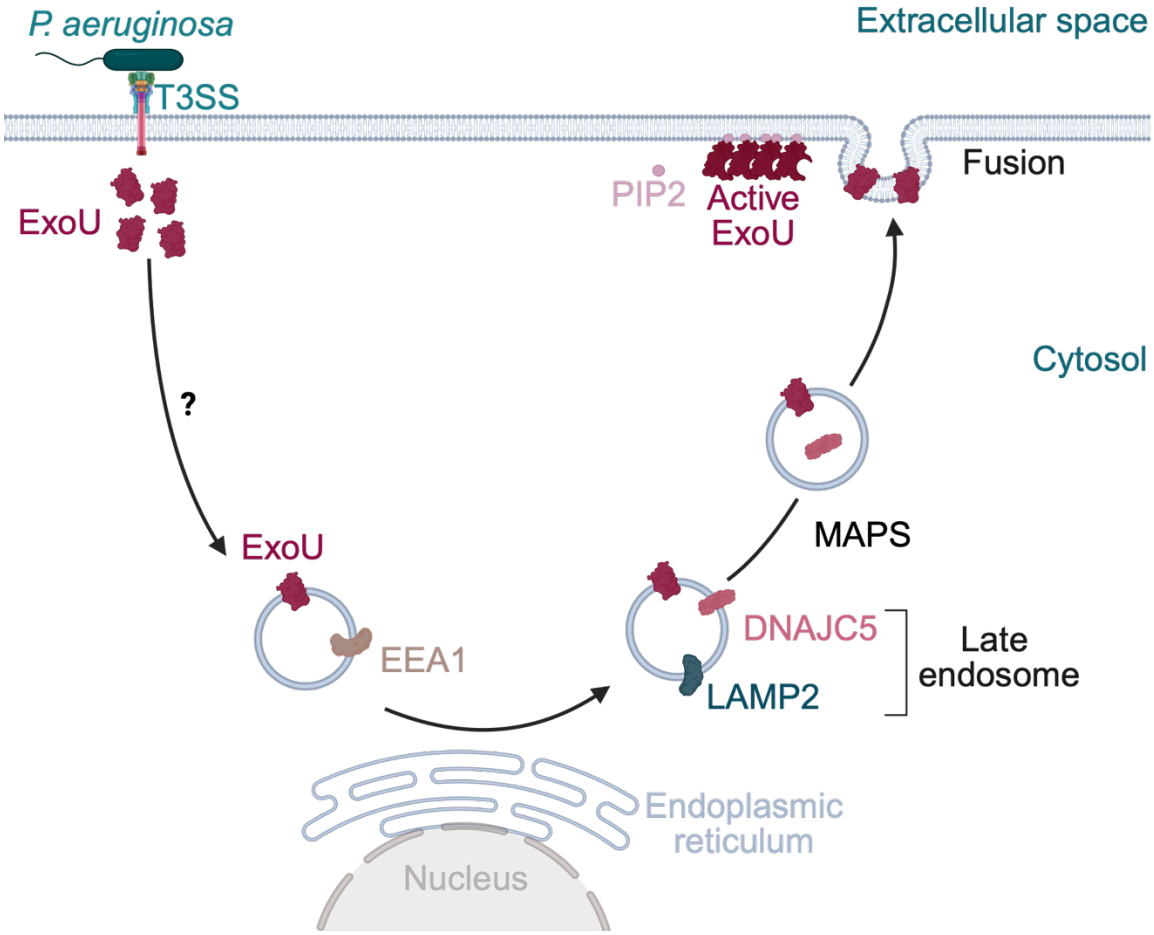

Figure 27. The intracellular journey of ExoU.
Upon injection, ExoU traffics probably through the endocytic pathway, as it localizes with EEA1 marker, to reach the perinuclear region. ExoU localizes to late endosomes (LE) decorated with LAMP2 and DNAJC5 proteins. The mechanism allowing ExoU to anchor the LE endosomes remain unknown. From there, ExoU traffics through the MAPS pathway to reach the PM. When the DNAJC5 vesicle fuses with the PM, ExoU binds its cofactor the PI(4,5)P2 leading to the activation of the phospholipase activity of ExoU and therefore PM rupture. According to (Deruelle et al., 2021). Made with Biorender.

Chapter 4 - Proximity labeling as a tool for proxisome mapping

Proximity labeling (PL) is an exponentially growing approach in the field of biochemical tools used for protein-protein interaction and identification of proteins found in proximity. It differs from the classical *in vitro* methods such as complexes purification through affinity capture and yeast/bacterial two hybrid systems by taking place *in vivo* within the natural environment of the protein of interest (POI) (Martell et al., 2012; Roux et al., 2012a). One approach to perform proximity labeling (PL) is through the use of proximity-dependent biotinylation (PDB) tools.

4.1. PDB tools and their evolution

4.1.1. The ascorbate peroxidase (APEX)

A first commonly used tool for PL is the engineered ascorbate peroxidase APEX of 28 kDa derived from the cytosolic plant ascorbate peroxidase (APX) (Martell et al., 2012). APEX is fused to the POI and expressed inside cells. The activity of APEX is stimulated upon the addition, for 1 min, of exogenous H_2O_2 and biotin-phenol. Then, APEX oxidizes the biotin-phenol leading to the generation of the unstable short-lived biotin-phenoxyl radical. This radical marks covalently the proteins that are in the close environment of the fusion protein APEX-POI. This biotin mark allows to enrich the tagged proteins on streptavidin beads and their subsequent identification by mass spectrometry (MS) (**Figure 28**). (S. S. Lam et al., 2015; Nguyen et al., 2020). Directed evolution of APEX yielded a more active and thermally stable version termed APEX2, with a single mutation « A134P » in comparison to APEX (S. S. Lam et al., 2015). Later on, split APEX (sAPEX) was engineered. It consists of an optimized N-terminal 200 amino acid (aa) fragment and a 50 aa C-terminal fragment. Each of these fragments could be individually fused to a target protein and when they co-proximate, the sAPEX is reconstituted and becomes active. This approach allows to reduce the background labeling originating from off-target peroxidase (Han et al., 2019). The labeling radius of APEX is estimated to be less than 20 nm (< 20 nm) (Nguyen et al., 2020) (**Figure 28**).

The employment of the APEX tool lead to the profiling of different subcellular proteomes, such as the interface between ER and mitochondria, mitochondrial intermembrane space and outer membrane (I.-T. Cho et al., 2017; Hung et al., 2016, 2017; Rhee et al., 2013). In addition, several proteomes were identified including the ones of lipid droplets, the neurodegenrative protein α-synuclein and disease-related stress granules (Bersuker et al., 2018; Chung et al., 2017; Markmiller et al., 2018). It is also possible to determine the subcellular localization of the POIs combined or not with electron microscopy such as nuclear, ER and mitochondrial proteins (S.-Y. Lee et al., 2016, 2017; Martell et al., 2012). Lastly, PL using APEX has been applied on lysosomes and EVs in neuronal cells (Frankenfield et al., 2020; L. Song et al., 2021) allowing the identification of novel and known EVs and lysosomes associated proteins in neuronal cells.

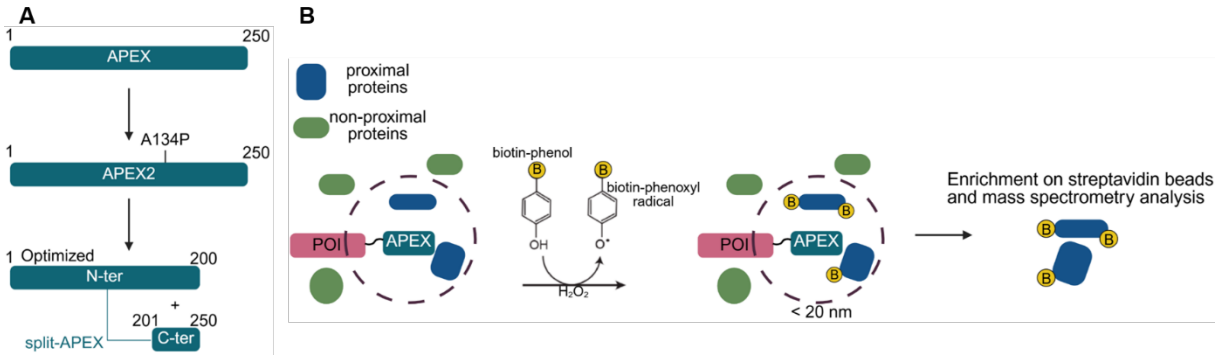

Figure 28. The evolution and mechanism of action of APEX as a proximity labeling tool.
(**A**). The evolutive process that began with APEX and allowed the development of the split-APEX. The indicated numbers are the amino acids position and A134P is the one residue difference between APEX and APEX2. (**B**). The mode of action of APEX. In the presence of exogenous H_2O_2 and biotin-phenol, APEX catalyzes the formation of the unstable biotin-phenoxyl radical and then biotin is transferred to the proteins that are proximal to the POI-APEX fusion within a 20 nm radius. Biotinylated proteins are subsequently analyzed by mass spectrometry. Made with Biorender.

4.1.2. The biotin identification (BioID)

In parallel to APEX, another PDB was described and developed. The proximity-dependent biotin identification (BioID) relies on the biotinylation activity of a biotin ligase. It started with the *E. coli* 35 kDa BirA biotin ligase (Chapman-Smith & Cronan, 1999). In the presence of ATP and exogenous biotin, the biotin ligase catalyzes the formation of a reactive intermediate biotinoyl-5'-AMP (bioAMP) which is then held within the active site of the biotin ligase. BioAMP is then released and reacts with nearby lysine residues on proximal proteins (Lane et al., 1964). The biotinylated proteins are thereafter enriched and analyzed in a similar manner as described for the APEX tool (Roux et al., 2012b). BirA was modified (R118G) to BioID (BirA*), a more promiscuous biotin ligase, which releases the reactive bioAMP faster than BirA (Kwon & Beckett, 2000) and is active within live mammalian cells (Roux et al., 2012b). Later, the biotin ligase from *Aquifex aeolicus* was humanized, mutated and tested for biotinylation in eukaryotic cells. This version, termed BioID2 (26.4 kDa), disrupted less the targeting/localization of the fusion protein and required lower biotin quantities compared to BioID (10 times less) yet both needed 16 h for maximal biotinylation (D. I. Kim et al., 2016). Similarly to APEX, split BioID (sBioID) was generated when BioID is cut into two fragments at the split site E256/G257 still requiring 16-24 h for biotinylation (Schopp et al., 2017) (**Figure 29**).

In order to reduce the biotinylation time, BirA was further submitted to directed evolution with over 29 rounds of stringent selection which yielded two new promiscuous versions : TurboID and miniTurbo (Branon et al., 2018). TurboID is of 35 kDa with 15 mutations relative to BirA and miniTurbo (28 kDa) is the BirA with the N-terminal domain deleted and 13 mutations. Both of them are able to efficiently biotinylate only in 10 min upon biotin addition (Branon et al., 2018). TurboID is the most active biotin ligase reported yet it has a strong background activity

while miniTurbo has half the activity of TurboID (Branon et al., 2018) and is unstable (May et al., 2020). Therefore, BioID2 was split at the site K171/S172 which corresponds to the sBioID site. The N-terminal fragment, 19.4 kDa, of BioID2 was termed microID and it showed a biotinylation activity at short labeling times. microID was then subjected to directed evolution leading to UltraID, the microID-R40G/L41P variant. UltraID biotinylates in a similar efficiency compared to TurboID (10 min) but with the advantage of a decreased background noise (Kubitz et al., 2022). The estimated labeling radius of BioID is ~10 nm (D. I. Kim et al., 2014) and it was shown that it could be increased by using a flexible linker between BioID and the POI (D. I. Kim et al., 2016) (**Figure 29**).

BioID and its derivatives have been applied in eukaryotic systems to investigate a wide variety of cellular processes and molecular contexts (Varnaitė & MacNeill, 2016). Few examples include the identification of the DNAJC5 proxisome in neuronal cells (E. Barker et al., 2024), mapping the human cell and defining intracellular location of proteins in HEK 293 cells (Go et al., 2021), better comprehend the human mitochondrial interaction network (Antonicka et al., 2020) and to identify, *in vivo*, synaptic proteins in mouse brain (Uezu & Soderling, 2019).

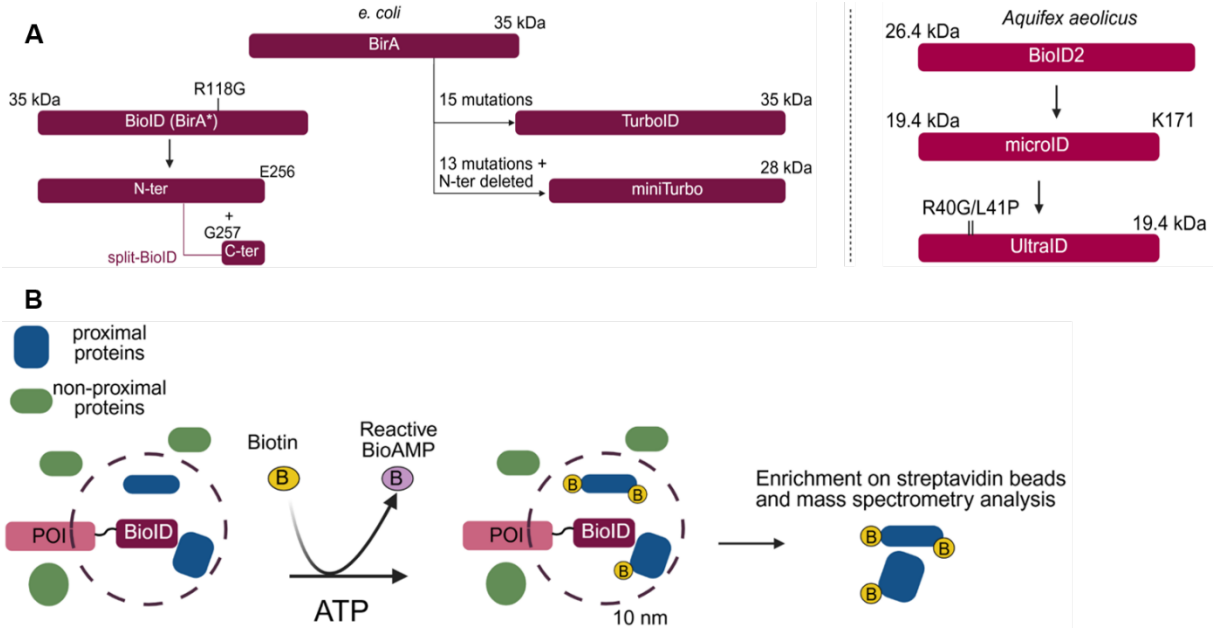

Figure 29. The evolution and mechanism of action of biotin ligase as a proximity labeling tool. (**A**). The evolutive process starting with the BirA (35 kDa) of *e. coli* or the BioID2 of *Aquifex aeolicus* (26.4 kDa). This allowed the development of the split-BioID and the obtention of a 19.4 kDa biotin ligase known as UltraID. The accumulated mutations from one generation to the other are indicated. (**B**). The mode of action of biotin ligase. In the presence of exogenous biotin, BioID catalyzes the formation of the reactive intermediate BioAMP (biotinoyl-5'-AMP) then biotin is transferred to the proteins that are proximal to the POI-BioID fusion within a 10 nm radius. Biotinylated proteins are subsequently analyzed by mass spectrometry. Made with Biorender.

4.2. Proximity labeling applied to bacterial proteins and effectors

PDB tools have been also applied on a variety of bacterial proteins. APEX2 application allowed a better understanding of the T6SS biogenesis of Gram-negative bacteria. The T6SS is a contractile tail structure allowing the injection of a syringe filled with effectors into target cells (bacteria or eukaryotic cells) (Basler et al., 2012; Brackmann et al., 2017; Durand et al., 2014). The tail includes the inner tube surrounded by the contractile sheath (Basler, 2015; Kudryashev et al., 2015). APEX2 was fused to the N-terminus of TssA , a protein involved at all stages of the assembly of the T6SS (Zoued et al., 2016), of the enteroaggregative *Escherichia coli* (EAEC) (Santin et al., 2018). PL followed by MS and then the use of several mutations that block the T6SS assembly at specific stages allowed the identification of a novel modulator of the T6SS. More precisely, TagA localizes to the distal end of the sheath, stops the polymerization of the sheaths and stabilizes the extended, prior to contraction, sheath conformation (Santin et al., 2018).

The nosocomial pathogen *Staphylococcus epidermis* is able to form biofilms (Costerton et al., 1999) which contain a major polysaccharide, the (1→6)-β-N-acetylglucosamine (PNAG) (McKenney et al., 1998). In a recent study, the interactions within its biofilm were investigated by trageting the proxisome of PNAG through an unusual approach. The mutant version of the glycosyl hydrolase enzyme Dispersin B (DspB), able to bind PNAG without hydrolyzing it, was fused to APEX2. The $DspB_{mut}$-APEX2 fusion was recombinantly expressed, purified and exogenously added on *S. epidermis* growing biofilms. This allowed to discover the proxisome of PNAG within the biofilm and identified the elastin-binding protein (EbpS) as a main interactor with PNAG specifically through its conserved lysin motif (Vo et al., 2023).

Chlamydia trachomatis (*Ctr*) is an obligate intracellular pathogen which grows within inclusions known as bacterium-contaning vacuole (BCV) (Brunham & Rey-Ladino, 2005; Moore & Ouellette, 2014). The T3SS of *Ctr* secretes several effectors including the class of Incs which have two transmembrane domains and are present on the inclusion membrane in contact with the host cytosol (Dehoux et al., 2011; Mueller et al., 2014). APEX2 fused to Incs identified potentially 400 proteins associated with the inclusions at different stages of the infection. In addition, an essential association between the inclusions and the ERES (endoplasmic reticulum exit sites) was highlighted (Dickinson et al., 2019). Another study on two Incs fused to APEX2, IncA and IncF, identified endogenous host factors localizing at the inclusion membranes : the leucine-rich repeat flightless-interacting protein 1 (LRRF1) and its binding partner, protein flightless 1 homolog (FLII) (Olson et al., 2019).

Another Chlamydia, *Chlamydia psittaci*, was investigated this time using BioID. The T3SS secreted protein SINC is observed at the host's nuclear envelope, is imported into the nucleus

and specifically targets the inner nuclear membrane. SINC was then fused to different versions of BioID which allowed to identify a set of 22 high confidence candidates that are mainly inner nuclear membrane proteins involved in chromatin silencing, nuclear signaling and structure (Mojica et al., 2015). This showed that *C. psittaci* targets the nuclear envelope through its SINC T3SS effector and paves the way for upcoming studies to better understand the importance of this interaction. In both cases, APEX2 and BioID, the fusion protein was expressed in Chlamydial cells.

Legionella pneumophilia is a facultative intracellular pathogen able to cause pneumonia and replicates within *Legionella*-containing vacuole (LCV) (Horwitz, 1983; Newton et al., 2010). It uses a T4SS that translocates effectors into host cells including the PieE protein (De Felipe et al., 2008; Ninio et al., 2009). It was shown that PieE is localized to the ER and the LCV in addition to reorganizing the ER. The expression, in host cells, of BirA fused to PieE allowed to identify 20 proteins with different functions in the trafficking pathways : vesicular transport between the ER and the Golgi, secretion at the ERES, trafficking in endosomal pathway and post-Golgi recycling and autophagy proteins (Mousnier et al., 2014) knowing that *Legionella* indeed does hijack and manipulate the corresponding pathways (reviewed in (Weber & Faris, 2018). Further analyses showed a direct interaction of PieE with several identified GTPases (Rab1a, 1b, 2a, 5c, 6a, 7 and 10) specifically through its two coil-coiled regions 1 and 2 unraveling new aspects of *Legionella* infection (Mousnier et al., 2014).

Five host intracellular trafficking manipulators of the T3SS of *S*. Typhimurium were fused to BioID and transiently expressed in the host cell (SifA, PipB2, SseE, SseG and SopD2) (D'Costa et al., 2019). This allowed to identify 381 possible interactions with human proteins related to cytoskeletal elements and vesicles trafficking and transport. Deeper analyses highlighted an interaction between SifA and the BLOC-2 complex (Biogenesis of Lysosome-related Organelles Complex 2) which plays a role in lysosome trafficking and function (K. Li et al., 2014). In this case, BLOC-2 is involved in the LAMP1 compartment aggregation induced by SifA, is essential for the modulation of the *Salmonella*-containing vacuole (SCV) positioning by SifA and lastly it decreases the stability of the SCV in the absence of the critical SifA (D'Costa et al., 2019). This shows once again that PL is a valuable tool to gain insights into host-pathogen interactions.

These examples demonstrate the broad use of PL tools to study interactions within bacteria or within host cells during host-pathogen interactions, where bacterial effectors are fused to the PL enzyme. They also pinpoint that mostly, the fusion protein (PL-POI) is transiently expressed in host cell and occasionally in the pathogen from where it is naturally secreted into the host. For instance, in Chlamydia, fusion proteins are efficiently exported across the T3SS needle

and translocated into host cells. Indeed, applying PL tools to bacterial effectors naturally injected into host cells remains challenging.

Results

Chapter 5 - Proxisome of the *Pseudomonas aeruginosa* Toxin ExoU Reveals Protective Roles of Host SLC3A2 and SNAP23

5.1 Publication

Proxisome of the *Pseudomonas aeruginosa* Toxin ExoU Reveals

Protective Roles of Host SLC3A2 and SNAP23

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In this work, Stéphanie Boullot performed most of the immunofluorescence experiments and their analysis in addition to the cytotoxicity and retraction data analysis by R studio

Vincent Deruelle performed the ExoY-UltraID injection assay

The mass spectrometry analysis was performed by Alwéna Tollec and Yohan Couté

I performed the rest of the experiments

Abstract

Pseudomonas aeruginosa (*Pa*) is a major human Gram-negative opportunistic pathogen able to cause a variety of infections. It harbors an arsenal of virulence factors including the potent Type 3 Secretion System (T3SS) which injects four toxins into eukaryotic cells. Among them, the most aggressive toxin, the phospholipase ExoU targets the host plasma membrane (PM) leading to a rapid necrotic cell death. Recently, our team identified the host chaperone DNAJC5, critical for the Misfolding-Associated Protein Secretion (MAPS) pathway, as essential for targeting ExoU to the PM. However, other host factors governing the intracellular journey of ExoU remain unknown. In this study, we aimed to better understand the crosstalk between ExoU and the host's trafficking machinery. We expressed ExoU fused to the biotin ligase UltraID in *Pa* and performed PL assays during infection. In the presence of exogenous biotin, UltraID mediates the biotinylation of proteins in close proximity to ExoU which are then analyzed by LC-MS/MS. Analysis revealed nine enriched human proteins when ExoU-UltraID is injected, among them SNAP23 and SLC3A2. Cells that lack either of these two proteins showed a specific increased sensitivity to an infection with $ExoU^+$ strain. In addition, immunofluorescence revealed a partial colocalization of ExoU with SLC3A2 and CD63, marker of extracellular vesicles (EVs). Given that SNAP23 mediates the exocytosis of CD63-positive EVs, we hypothesized that ExoU is detoxified *via* this pathway. Consistent with this, ExoU was detected in isolated EVs from the supernatant of infected cells. For the first time, we applied PL to a T3SS injected toxin of an extracellular pathogen. We identified host factors taking part in the host cell response to ExoU, underlining a complex host-ExoU interplay.

Introduction

Bacterial infections are a current major health concern, due to the lack of vaccines and more importantly to the rising antimicrobial resistance (AMR). In 2021, 4.71 million deaths were estimated to be associated with bacterial AMR (1). As reported by Ikuta et al., 13.7 million deaths were infection related in 2019, 54.9% of which caused by 5 bacterial organisms: *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (*Pa*) (2). During colonization, bacteria manipulate their host in order to escape the immune and cellular responses induced upon infection, using a panoply of toxins and effectors (3–5). Whereas classical toxins are released from bacteria in the extracellular milieu, the so-called effectors are exported from the bacteria into host cells through complex secretion nanomachineries assembled at the bacteria surface (6). Within the eukaryotic host cytoplasm, the effectors target or exploit cellular pathways to promote pathogen uptake and infection (7). Overall, host-pathogen interactions are quite complex, as the outcome depends on the cumulative effects of multiple, often opposing, factors at play.

Pa is a major human Gram-negative nosocomial pathogen able to cause a variety of infections such as wound, bloodstream, urinary and respiratory tract infections (8). It is a multidrug-resistant organism, part of the ESKAPE pathogens that have been classified by the World Health Organization (WHO) as high priority pathogens for which novel antibiotics are urgently required (9,10). *Pa* can take advantage of breaches in hosts' barriers (skin, epithelium), of immunosuppressed patients or of altered lung environment in cystic fibrosis (CF) patients (11). It owes its virulence and pathogenicity to the arsenal of virulence factors and appendages encoded by its genome (12). *Pa* harbors on its surface a variety of secretion systems including the Type 3 Secretion System (T3SS), which promotes bacterial invasion and infectivity (13). The T3SS, identified as a potential anti-bacterial target, is a multiprotein needle-like apparatus that injects toxins into eukaryotic cells (14). In *Pa*, four effectors are injected by the T3SS: ExoS, ExoT, ExoY and ExoU. ExoS and ExoU are usually mutually exclusive in a given *Pa* strain (14). ExoS and ExoT are 75% identical and both carry an ADP ribosyl transferase

(ADPRT) and a GTPase-activating protein (GAP) activities (15). ExoY is a promiscuous nucleotidyl cyclase with a broad substrate specificity (16). The T3SS effectors have been extensively studied and host co-activators have been identified for each: F-actin for ExoY (17), 14-3-3 adaptor protein for ExoS and ExoT and ubiquitin and inner membrane lipid phosphatidylinositol-4,5-bisphosphate PI(4,5)P2 for ExoU (18–20). ExoU is the most potent T3SS toxin with a phospholipase A2 (PLA2) activity damaging the host plasma membrane (PM) leading to a rapid cell death (21). ExoU is prevalent in aggressive *Pa* clinical strains and its presence is associated with poor prognosis and clinical outcome (22).

The trafficking of many bacterial toxins has been investigated and they often take advantage of host machineries to exert their activity and confer an advantage to the pathogen (7,23). ExoS localizes to the PM and late endosomes (LE) and can alternate between retrograde and anterograde trafficking pathways from and to the PM, respectively (24). ExoU is known to localize with the early endosome antigen 1 (EEA1), marker of early endsomes (EE) (20). In a recent CRISPR-Cas screen on A549 lung epithelial cancer cells, our team identified the host chaperone DNAJC5/CSPα as an essential host factor for ExoU-dependent cell death (25). DNAJC5 takes part in the MAPS - Misfolding associated protein secretion- pathway (26) and ExoU localizes with DNAJC5 vesicles (25). Yet no direct interaction between DNAJC5 and ExoU could be evidenced and other host factors involved in the ExoU journey within the eukaryotic cell remained unknown.

Proximity labeling (PL) is a technology that has been developed to determine not only the interactants of a protein of interest (POI) but also proteins localized within the same cellular compartments at a given time point (27). Biotin ligase (BL) and ascorbate peroxidase (APEX) are commonly used as PL enzymes and are fused to the POI. This approach is based on the fact that upon addition of the exogenous substrate, the enzymes are activated and biotinylate proximal proteins. The biotinylated proteins are enriched and subsequently analyzed by mass spectrometry (MS) (28–30). PL enzymes have been widely applied in eukaryotic systems to investigate different cellular processes (31,32). On the other hand, few host-pathogen

interactions have been investigated for intracellular bacterial pathogens either by transient transfection of the POI-enzyme fusion in eukaryotic cells (33) or by expressing the fusion within the pathogen during infection (34).

In this study, we applied PL on *Pa* effector ExoU during eukaryotic cell infection to get insights into ExoU's host proxisome. PL followed by LC-MS/MS analyses identified nine human proteins in the proximity of ExoU. Cells that are knocked-out (KO) for either of the two top hits, SLC3A2 or SNAP23, were more sensitive to ExoU compared to their respective wild-type (WT) background. Immunofluorescence and extracellular vesicles (EVs) isolation assays point toward a role for SLC3A2 and SNAP23 in the host's response to ExoU-dependent *Pa* cytotoxicity highlighting a complex host cell hijacking strategy of this toxin.

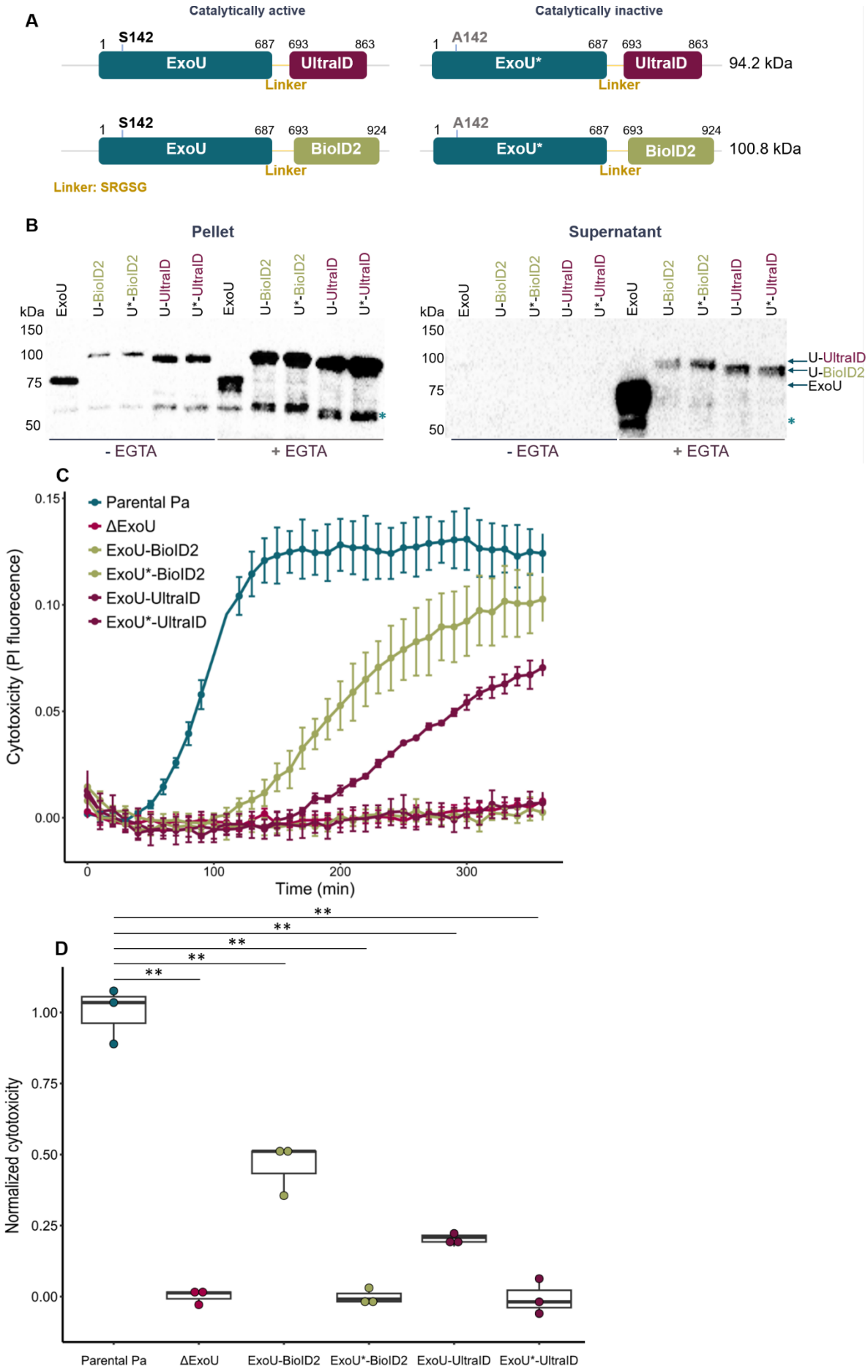
Results

ExoU-UltraID is secreted, translocated and active

Several BLs exist including BioID2 and UltraID. BioID2 derives from *Aquifex aeolicus* while UltraID is the optimized N-terminus of BioID2 (35), both with a biotinylation radius of 10 nm (35,36). To perform the PL experiments, we fused the C-terminus of ExoU to UltraID and BioID2, with a linker in between (Fig 1A). We constructed both the active ExoU and the catalytically inactive ExoU* in which the amino acid serine at position 142 is replaced by alanine (S142A) (37). The expression and the secretion, in a Δ*exoU* background strain, of each construct was verified first *in vitro* as the T3SS needle is a thin channel with an inner diameter of 2-2.5 nm (38). In addition, T3SS effectors must be properly unfolded by the bacterial ATPase prior to the release into the needle (39,40). Calcium (Ca^{2+}) chelation by EGTA was used to induce the secretion by the T3SS *in vitro* (34) and ExoU presence was assessed in bacterial pellet and supernatant fractions using immunoblotting. We observed the expression of ExoU-BioID2/UltraID within the bacteria in addition to their secretion in a T3SS-dependent manner in the culture supernatant, yet in less quantities compared to the parental strain secreting ExoU (Fig 1B).

We then investigated the delivery of fusion proteins into A549 WT epithelial cells by monitoring cell death caused by the phospholipase activity of ExoU. We use the active ExoU forms to confirm that the fusion proteins are well injected, monitoring target cells death by previously established assays using propidium iodide (PI) incorporation as a proxi of *Pa* ExoU-dependent cytotoxicity (25), whereas the inactive forms were used to conserve the eukaryotic host of interest during the proximity labeling assay (PLA). Both strains harboring ExoU-UltraID and ExoU-BioID2 were cytotoxic on eukaryotic cells meaning that ExoU-fusions are translocated into the host cells. The cell death induced by the action of ExoU-BioID2 was faster than the one of ExoU-UltraID, albeit both were delayed compared to the cytotoxicity observed with the unmodified ExoU of the parental *Pa* that reached 100% of cell death at ~120 minutes (Fig 1C). ExoU-BioID2 and ExoU-UltraID reached half of the maximum PI signal of the parental *Pa* at 200 and 300 minutes respectively (Fig 1C). Altogether, these results show that ExoU fused to PL enzymes is less effectively handled by the T3SS machinery but are injected into target cells. In order to have an unrelated ExoU fusion protein as a negative control in the (PLAs), we generated ExoU fused to the improved fluorescence activating and absorption shifting tag (iFAST, (41,42) that was validated in a similar manner for secretion and cytotoxicity (Fig S1ABC).

For comparative and statistical analysis in the following work, the PI fluorescence data obtained from infected A549 WT cells was normalized to the maximum PI fluorescence signal of the parental *Pa* strain and presented as the area under the curve (AUC) of each condition (Fig 1D). We observe a similar result to the cytotoxicity curves in (Fig 1C) with the conclusion that ExoU fusions are injected to a lesser extent compared to the parental *Pa*. As the native ExoU requires DNAJC5 to induce cell death (25), we verified whether ExoU fusions similarly provoke cell death in a DNAJC5-dependent manner. Cytotoxicity assays performed on A549 cells KO for DNAJC5 (DNAJC5$^{-/-}$) showed the requirement of DNAJC5 for cell death, indicating that the BioID2, UltraID (Fig 1E) and iFAST (Fig S1D) fusions use the same trafficking as the parental *Pa* injecting the native ExoU.


A
Catalytically active
Catalytically inactive
S142
A142
ExoU
ExoU*
UltraID
BioID2
Linker
94.2 kDa
100.8 kDa
Linker: SRGSG
B
Pellet
Supernatant
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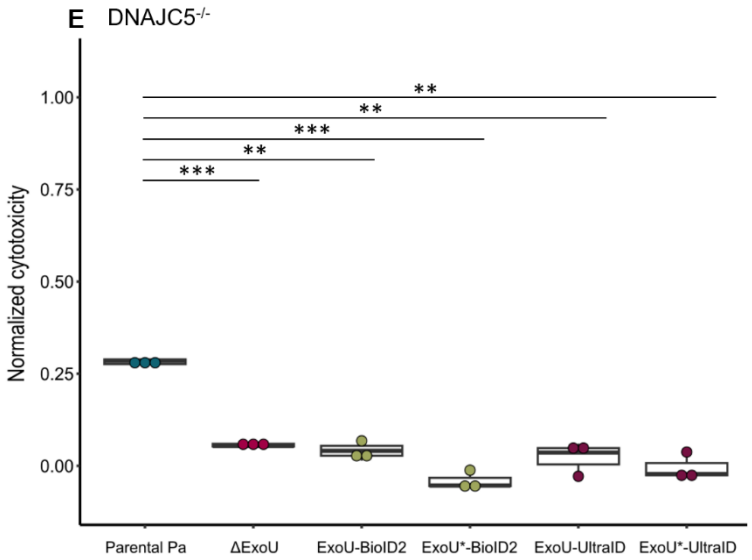


Fig 1. Engineered ExoU fusion proteins are secreted, active and require DNAJC5. A. Schematic representation of the fusion proteins constructs inserted into the genome of a Δ*exoU* strain. Active ExoU (S142) and inactive ExoU* (A142) fusions were generated. A linker (SRGSG) separates ExoU and the biotin ligases. **B.** Western blot showing the expression of the fusion proteins and their secretion in the supernatant upon an *in vitro* induction of the T3SS (+EGTA) compared to the unmodified ExoU. Western blot was revealed with α-ExoU antibody. ExoU degradation product is indicated by an * **C.** Cell death of A549 WT cells upon infection with ExoU-BioID2 and ExoU-UltraID expressing strains compared to the parental and ΔExoU *Pa*. Cytotoxicity is represented as mean ± standard deviation (SD) of PI fluorescence signal over time. **D.E.** Normalized cytotoxicity represented as the area under the curve (AUC) of A549 WT and DNAJC5$^{-/-}$ cells infected with different *Pa* strains.

We also applied the same strategy on the three other T3SS toxins ExoS, ExoT and ExoY by fusing them to UltraID (Fig S2A). We expressed them individually in a PAO1 strain devoid of ExoS, ExoT and ExoY (PAO1Δ3tox) (Fig S2A). To verify the injection into host cell of each of the fusion proteins; cells rounding for the ExoS and ExoT fusions (Fig S2B) and host cGMP production for the ExoY fusion (Fig S2C) were monitored during infection. All three fusions were injected into host cell in a delayed manner compared to the parental *Pa*.

We moved forward and performed PLAs followed by MS analysis. Despite that all four fusions were injected into the host, only the ExoU*-UltraID fusion was significantly detected and will be further presented here.

PL followed by proteomics identify nine human candidates within ExoU proxisome

We performed a primary biotinylation assay by infecting A549 WT cells with the *Pa* strain injecting ExoU*-UltraID or ExoU*-BioID2 in the absence or presence of 500µM of biotin. We biotinylated for 1 hour with the ExoU*-UltraID strain and for 18 hours with the ExoU*-BioID2

strain. The cells were lysed (input) and the lysate was incubated with streptavidin beads to capture biotinylated proteins (elution). The analysis of the samples by Western blot using streptavidin-HRP molecule revealed a clear enrichment of biotinylated proteins in the elution of the ExoU*-UltraID conditions, compared to the input. In addition, a faint signal that could correspond to the molecular weight of the auto-biotinylated ExoU*-UltraID of ~100 kDa was detected (Fig 2A). The other proteins revealed in similar quantities in the conditions with and without biotin, migrating between 50 and 75 kDa, probably represent endogenously biotinylated eukaryotic and bacterial proteins, such as biotin-dependent carboxylases and LiuD, respectively (43–45). As the assay with ExoU*-BioID2 failed to enrich biotinylated proteins nor showed the auto-biotinylated fusion protein (Fig 2A) we focused on the UltraID condition and further optimized it by increasing the time of biotinylation to 5.5 hours during infection. When revealing with streptavidin-HRP, we observed an important enrichment of the protein of ~100 kDa, which was confirmed to be ExoU-UltraID using specific α-ExoU antibodies (Fig 2B).

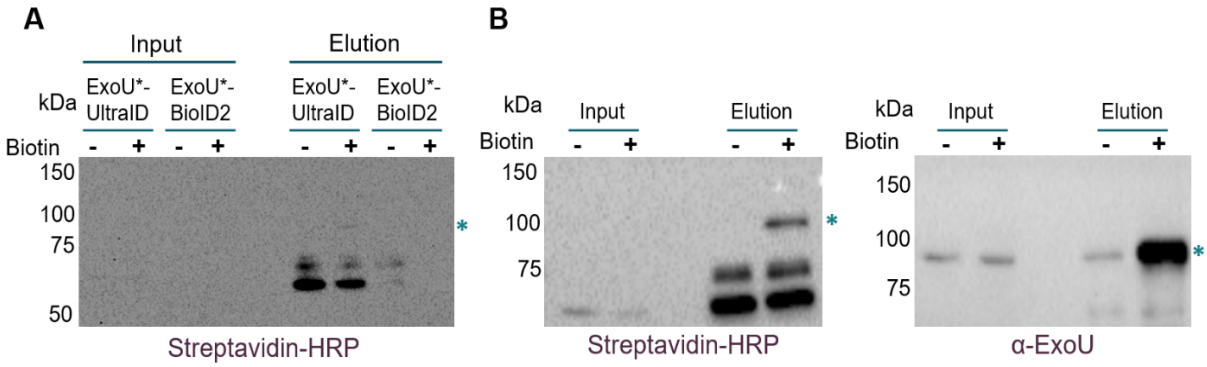


Fig 2. First proximity labeling assay (PLA) favors the use of ExoU*-UltraID strain and reveals auto-biotinylated ExoU*-UltraID. A. Western blot was revealed with streptavidin-HRP showing the enrichment of biotinylated proteins after PLA using A549 WT cells. Cells were infected either with the ExoU*-UltraID or ExoU*-BioID2 *Pa* strain and biotin was added (+) or not (-) at 500 µM. Biotin was applied for 1 hour with ExoU*-UltraID strain and for 18 hours with the ExoU*-BioID2. **B.** Western blot was revealed with streptavidin-HRP and with α-ExoU showing the enrichment of biotinylated proteins after PLA using A549 WT cells. Cells were infected either with the ExoU*-UltraID and biotin was added (+) or not (-) at 500 µM. Biotin was applied for 5.5 hours during infection. Auto-biotinylated ExoU*-UltraID ~100 kDa is indicated by an *.

Previously described and optimized conditions using ExoU*-UltraID were applied to perform the PL assay in triplicates followed by the identification of enriched proteins by LC-MS/MS. ExoU*-iFAST was used as a negative control for the ExoU toxin fused to an unrelated protein tag. The experiment was performed by infecting A549 WT cells ($2x10^7$ cells, MOI 100) and the

proteins captured by the streptavidin beads were subjected to LC-MS/MS. The MS detected altogether 2456 proteins, both of human and bacterial origin (Supplementary file 1). Proteins were considered enriched in the UltraID condition with a threshold of -log10(p_value) >2 and a log(FC) ≥ 1). It is to note that ExoU*-UltraID is one of the most enriched proteins compared to the iFAST condition, with a log(FC) of 6.58 and –log10(p_value) of 4.95, confirming biotinylation-dependent protein enrichment (Supplementary file 1).

The data of eukaryotic proteins present in three independent replicates is represented in a volcano plot as –log10(p_value) in function of the log(FC). In the iFAST condition, we observe proteins that bind nonspecifically to the non-saturated beads in the absence of UltraID (Fig 3). Conversely, analysis of MS data revealed nine human proteins specifically and significantly enriched in the ExoU*-UltraID condition: RAB27B, SNAP23, SLC3A2, STIP1, DCUN1D4, ERBIN, SPTBN1, SLK and ITGA3 (Fig 3). Using genecards (https://www.genecards.org/) available information and bibliography, we examined the pathways that the nine proteins have been associated with (Table 1). The most represented pathways were signaling by Rho GTPases and vesicles transport and trafficking, hinting towards novel actors in the ExoU intracellular voyage.

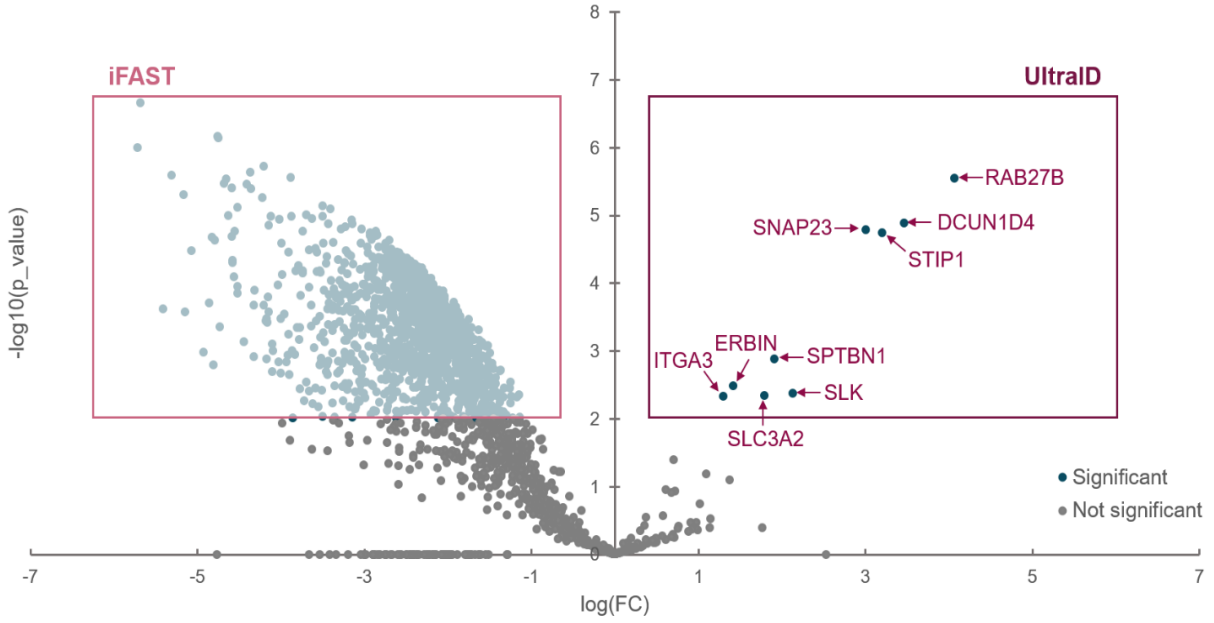


Fig. 3. PLA on A549 WT cells infected ExoU*-UltraID *vs* ExoU*-iFAST strains identifies nine host proteins of interest. Mass spectrometry analysis in triplicates of A549 WT cells infected with the ExoU*-UltraID or iFAST *Pa* strain. Data is represented as a volcano plot showing the –log10(p_value) in function of the log fold change (FC). Proteins are considered significantly enriched in the UltraID condition at a threshold of –log10(p_value) > 2 and a log(FC)>1 whereas proteins with a –log10(p_value)

> 2 and a log(FC) < -1 are significantly enriched in the iFAST condition. This cut-off leads to a FDR inferior to 3 % for each comparison according to the Benjamini-Hochberg estimator. Only human proteins are shown.

Protein	Pathway
RAB27B	Vesicle mediated transport, Rab regulation of trafficking
DCUN1D4	Metabolism of proteins
STIP1	Cellular responses to stimuli, signaling by Rho GTPases
SNAP23	Vesicle mediated transport, signaling by Rho GTPases, ER-Phagosome pathway
SLK	Signaling by Rho GTPases, RHOC GTPase cycle
SPTBN1	Transport to the Golgi and subsequent modification, MAPK family signaling cascades, signaling by Rho GTPases
ERBIN	Signaling by Rho GTPases, RHOC GTPase cycle, RAC2 GTPase cycle
SLC3A2	Transport of inorganic cations/anions and amino acids/oligopeptides
ITGA3	Integrin pathway, ERK signaling

Table 1. Biological pathways in which the nine eukaryotic proteins of interest are involved. Validated or predicted pathway(s) are taken from genecards (https://www.genecards.org/). The pathways with highest confidence score were considered.

To corroborate the previous MS data, we constructed and performed PL experiments with a *Pa* strain secreting but not injecting ExoU*-UltraID. This *Pa* strain lacks the two bacterial proteins PopB and PopD which are the effector translocators, located at the tip of the T3SS (46). We used the available strain with a Δ*popBD* genotype in a PAO1Δ3tox background. As PAO1 is a strain that does not naturally harbor ExoU, we generated a new construct containing ExoU*-UltraID in addition to the bacterial chaperone of ExoU, SpcU (see M&M). In the same experiment, we took advantage of the A549 DNAJC5$^{-/-}$ cells and compared the results to the ones obtained with A549 WT cells. Biotinylation assay resulted in the similar pattern of biotinylated proteins when revealed with streptavidin-HRP followed by α-ExoU, as previously shown (Fig S3). The PL was performed in triplicates and the enriched proteins were identified by LC-MS/MS. The comparison of A549 WT cells infected with the parental *Pa* ExoU*-UltraID *vs* Δ*popBD* ExoU*-UltraID showed that the previous hit, SLC3A2, is significantly enriched in the parental *Pa* condition when looking at the normalized abundances from the three replicates (Fig 4A). RAB27B was not detected (abundances were imputed) in the Δ*popBD* condition, whereas it was detected in the triplicates of the parental *Pa* condition yet with a large variance therefore showing no statistical significance (Fig 4A). Interestingly, the analyses of the data for SLC3A2 and RAB27B in the conditions of A549 WT *vs* A549 DNAJC5$^{-/-}$ infection, showed that

both are significantly enriched in the A549 WT compared to the DNAJC5$^{-/-}$ (Fig 4B). This suggests that SLC3A2 and RAB27B are associated with the intracellular journey of ExoU and in a DNAJC5-dependent manner.

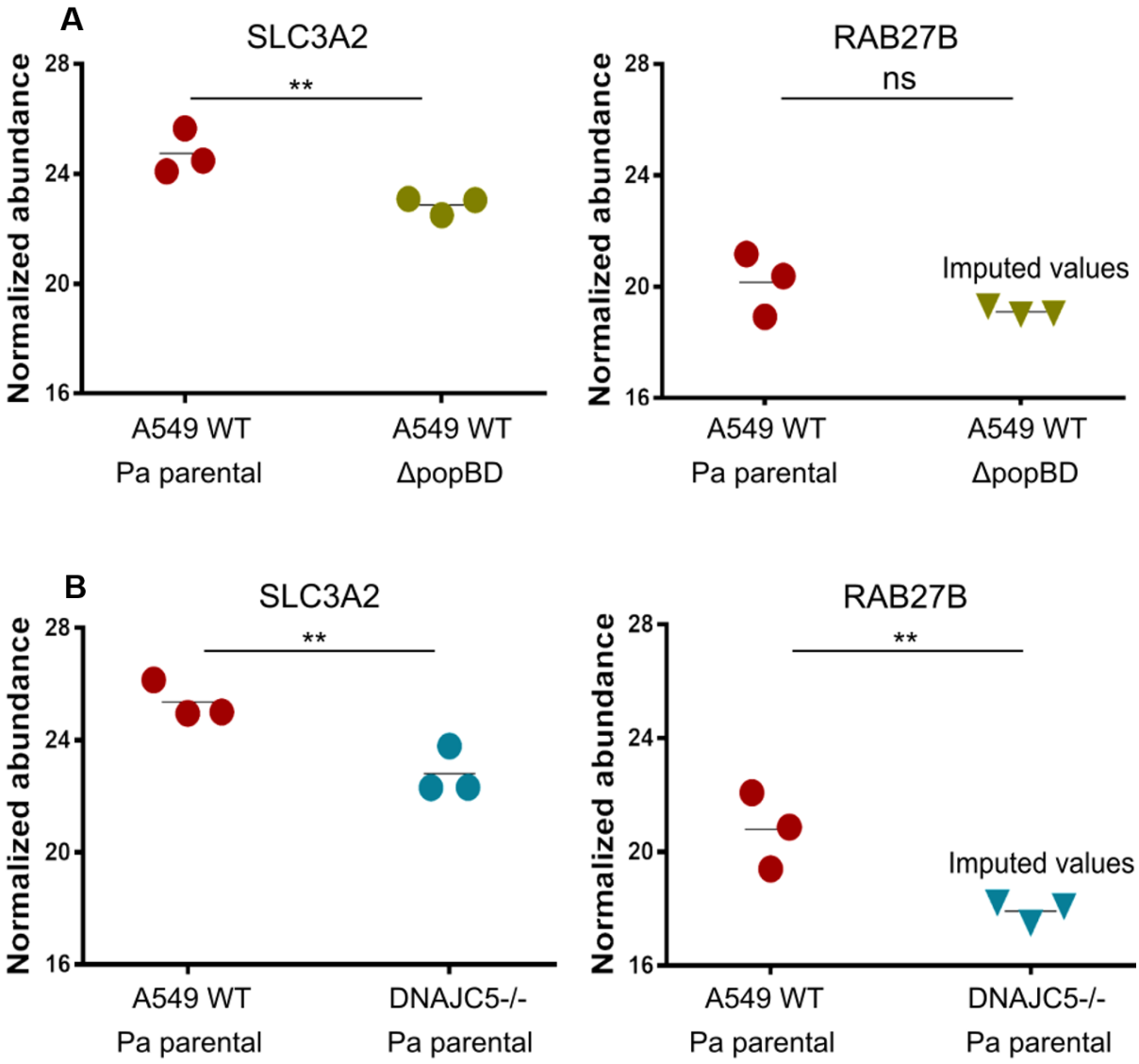


Fig 4. Translocation mutant (Δ*popBD*) and DNAJC5$^{-/-}$ cells confirm conserved hits and reveal specificities of the ExoU proxisome. A. Normalized abundances of SLC3A2 and RAB27B obtained by LC-MS/MS analysis of the triplicates of the PLA of two conditions: A549 WT cells infected either with parental or Δ*popBD Pa* strain. **B**. Normalized abundances of SLC3A2 and RAB27B obtained by LC-MS/MS analysis of the triplicates of the PLA of two conditions: A549 WT or DNAJC5$^{-/-}$ cells infected with the parental *Pa* strain. Missing values were imputed (triangles) to allow statistical analysis. ** : p-value < 0.01, ns: non-significant.

Investigation of a potential role of SLC3A2, RAB27B and SNAP23 in ExoU cytotoxicity

We focused our work on SLC3A2, RAB27B and SNAP23 as they have been proposed to participate in secretion in general and/or specifically to DNAJC5-dependent secretion

pathways. SLC3A2, known also as CD98, is a subunit of a heterodimeric amino-acid transporter (47). In a recent work, it was shown to interact with the linker domain of DNAJC5 and to be required for its perinuclear localization in the context of MAPS (48). RAB27B is a member of the Rab GTPase family and it regulates protein secretion at the transport and docking steps (49). It was demonstrated that RAB27B has a role in the release and clearance of α-synuclein (α-syn) in a DNAJC5- and MAPS-independent way (49). Although SNAP23 was only differentially detected in the ExoU*-UltraID *vs* ExoU*-iFAST triplicates (Fig 3), it was taken into consideration for its role in exocytic pathways and the extracellular release of different molecules (50). Moreover, it was shown that SNAP23 is required for the DNAJC5-stimulated extracellular release of neurodegenerative proteins such as tau and α-syn (50).

In the attempt to confirm these three hits, we first performed knock-down (KD) experiments using siRNAs as previously described (25) to evaluate the importance of each protein in ExoU-dependent cytotoxicity. Non-targeting siRNA was used as a negative control and two siRNAs per target were used against SLC3A2, RAB27B and SNAP23 individually or all together (Triple KD). Target proteins' expression was evaluated on days 1, 2 and 3 post-transfection compared to non-transfected (control) cells by Western blot using the respective antibody (Fig 5A). We observed a successful, yet not complete, down-regulation of the corresponding proteins over the course of 3 days post-transfection (Fig 5A). The effect of the KD on the cytotoxicity of ExoU was evaluated by infecting transfected cells with PA14 ($ExoU^+$) strain and the cytotoxicity was monitored as previously described. The cells that were knocked-down for SLC3A2 or for the three targets (triple KD) were more sensitive to the ExoU activity compared to the control (Fig 5B). We also used siRNAs to KD DNAJC5 and surprisingly, the DNAJC5 KD was barely able to alter the ExoU-dependent cytotoxicity, in disagreement with the requirement of DNAJC5 for ExoU activity (25). In addition, as RAB27B, SNAP23 and SLC3A2 siRNAs lowered respective proteins' amounts, the DNAJC5 downregulation was probably successful yet we could not verify it because the basal level of DNAJC5 in A549 WT cells was undetectable. Altogether, these results suggest that the KD, instead of KO (25), of a host factor is not sufficient to

evaluate its involvement in the ExoU-dependent cytotoxicity. Therefore, to conclude on the implication (or not) of the proteins of interest, we opted to test KO cell lines where their expression is completely abolished.

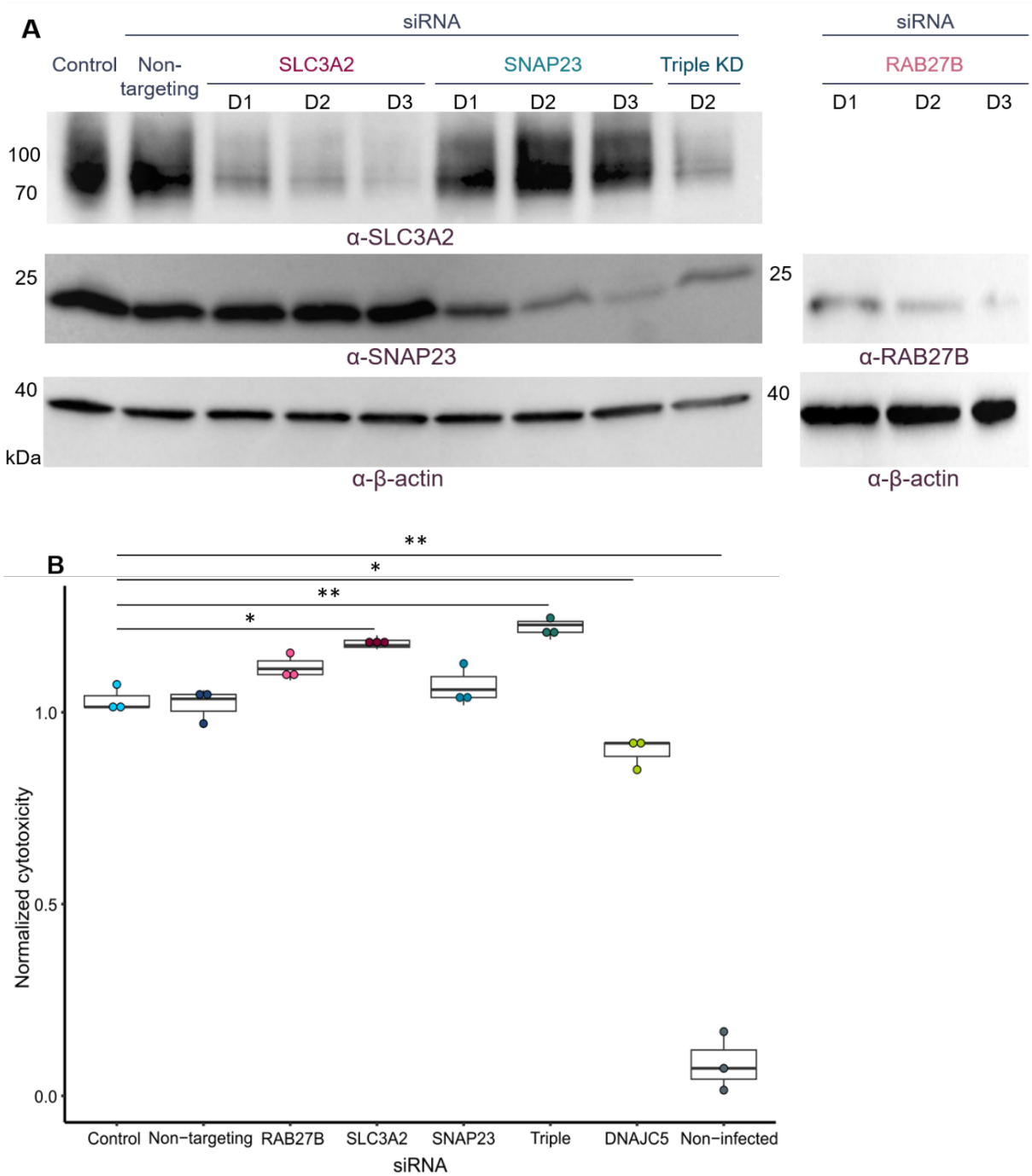
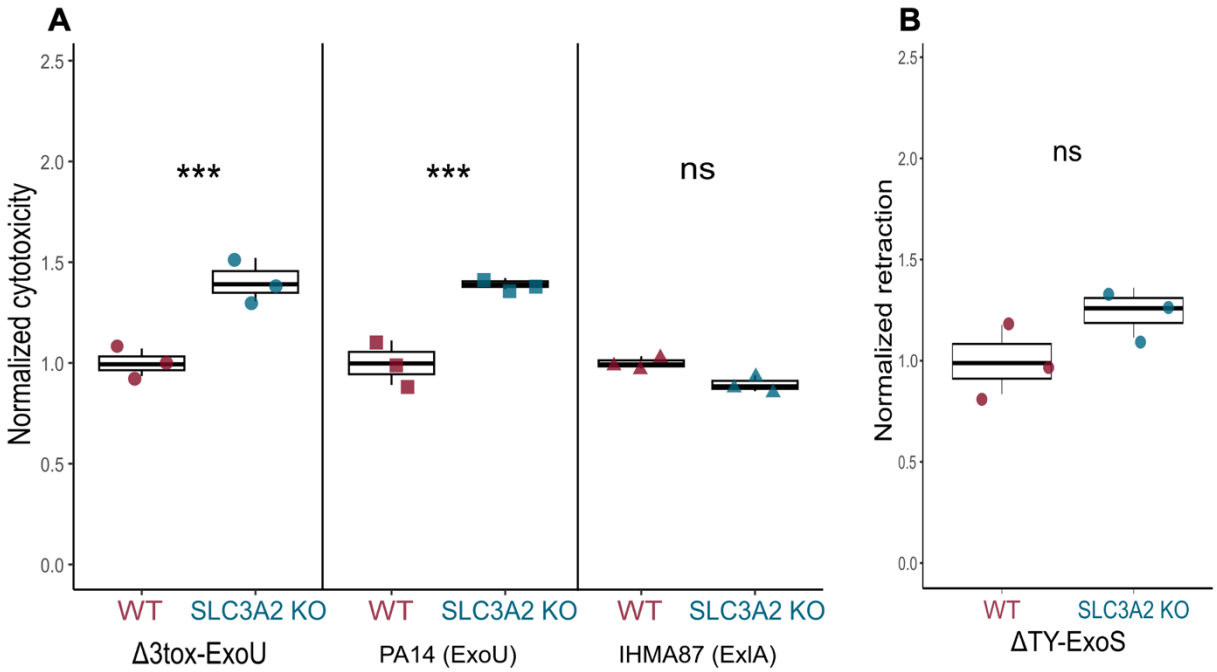


Fig 5. Knock-down (KD) of SLC3A2, SNAP23 or RAB27B does not affect the cytotoxicity of ExoU. **A.** A549 WT cells were transfected or not (control) with siRNAs for SLC3A2, SNAP23, RAB27B or the three targets at a time (triple KD) to KD their expression. A non-targeting siRNA was used as a control. KD was monitored by Western blot at days 1, 2 and 3 post transfection (D1, D2 and D3) and revealed with the respective antibodies. Actin was used as a loading control. **B.** Normalized cytotoxicity represented as the area under the curve (AUC) of siRNA transfected A549 cells infected with PA14 (ExoU$^+$) strain at 2 days post transfection (MOI 20). A549 WT cells transfected with siRNAs for DNAJC5 were used as a control.

SLC3A2 limits ExoU-dependent cytotoxicity

SLC3A2 KO dermal murine cells were obtained from C. Féral lab, Nice (51). These cells were used to study SLC3A2 in the context of intracellular *Brucella* infection. It was shown that SLC3A2 is required for an optimal bacterial uptake and intracellular replication (51). We compared the susceptibility of KO cells to the WT fibroblasts using two *Pa* strains expressing ExoU: a PAO1 strain lacking ExoS, ExoT and ExoY and expressing ExoU-ScpU (Δ3Tox-ExoU) to limit the effect of other T3SS effectors and a naturally ExoU-expressing strain, PA14. The AUC of the normalized cytotoxicity was significantly higher for the SLC3A2 KO cells compared to the WT cells upon infection, suggesting that SLC3A2 somehow limits the effect of ExoU activity (Fig 6A). To assess the global susceptibility of SLC3A2 KO cells to bacteria infection, we used the *P. paraeruginosa* IHMA87 strain lacking all T3SS effectors, but synthetizing another PM-damaging toxin Exolysin, ExlA (52). The cell lines showed no difference in the sensitivity to ExlA activity in the absence of SLC3A2 (Fig 6A), indicating that the SLC3A2-deficient cells do not present a global higher susceptibility to bacteria, or to the activity of PM-



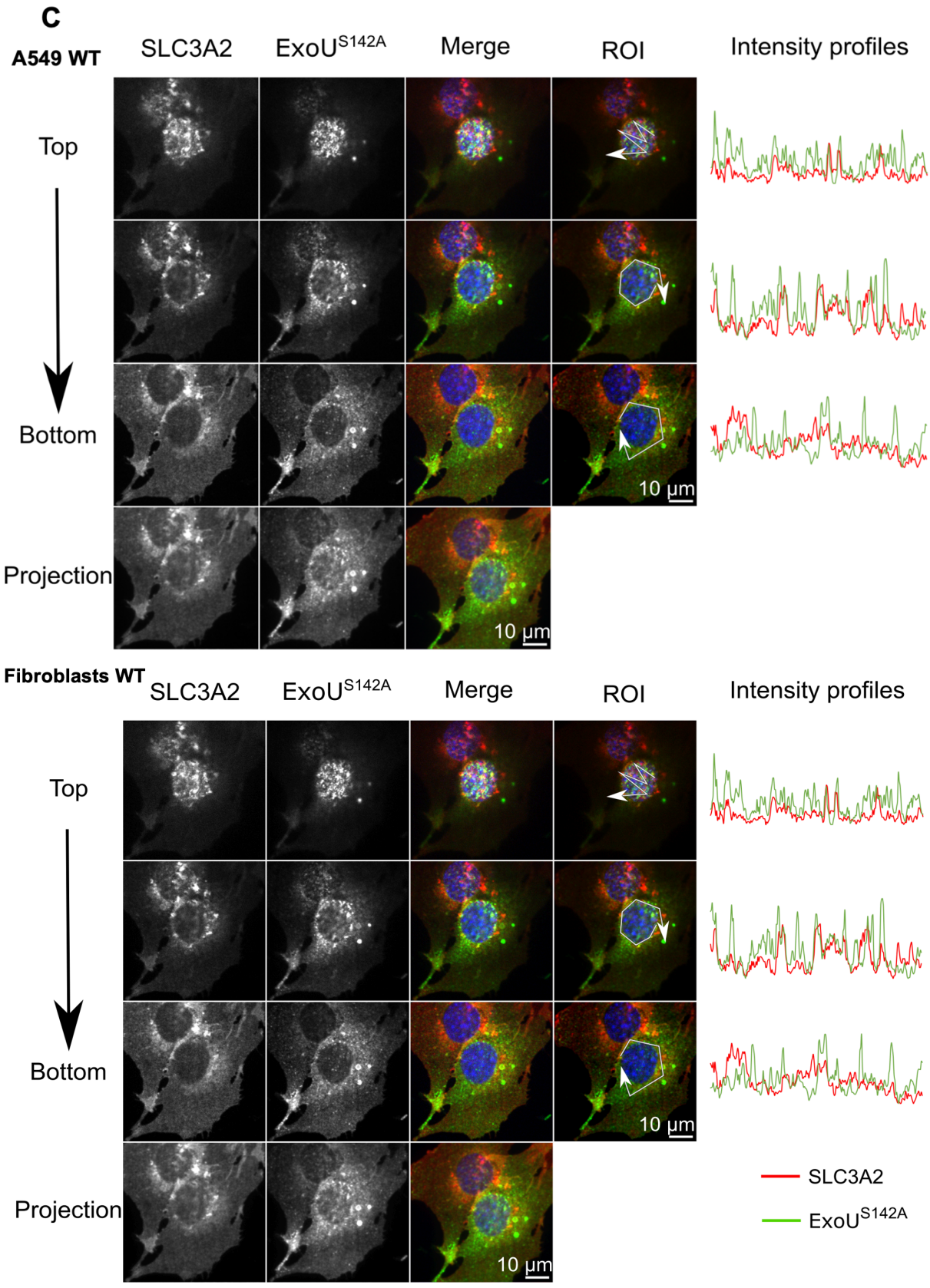


Fig 6. SLC3A2 controls ExoU-induced cell death. A. WT or SLC3A2 KO dermal murine fibroblasts infected with *Pa* strains Δ3tox-ExoU, PA14 or IHMA (MOI 0.1). Cell death was monitored by live-cell imaging and quantified by PI-positive cells over time. Data were normalized and the area under the curves (AUC) was calculated for each condition. WT cells are shown in red, SLC3A2 KO cells in blue and individual values are represented as dots. **B**. WT or SLC3A2 KO dermal murine fibroblasts infected with ΔTY-ExoS *Pa* strain (MOI 0.1). Cell retraction was monitored by live-cell imaging and quantified by

the number of retracted cells over time. Data were normalized and the area under the curve (AUC) was calculated for each condition. ***** :** $p < 0.001$; ns: not significant $p \geq 0.05$. **C**. A549 WT cells were infected for 4 hours with *Pa*-ExoU^{S142A} and stained for human SLC3A2 (red, shown in grayscale) and ExoU (green, shown in grayscale). Murine fibroblasts WT were infected in the same conditions and stained for murine SLC3A2 and ExoU. Each panel shows single Z-planes (top, middle, bottom) and a Z-projection. Columns display from left to right: SLC3A2 staining (grayscale), ExoU staining (grayscale), merge, merge with a manually selected region of interest (ROI) used for quantification and intensity profiles

damaging toxins. Finally, we tested also the SLC3A2-dependency for ExoS activity, which was measured by the level of cellular retraction that occurs due to disassembly of actin network upon ExoS enzymatic activities (53). The susceptibility to ExoS activity was not significantly different between the two cell lines (Fig 6B), denoting again an ExoU specific phenotype. The ExoU sensitive phenotype was independent of the used MOI (0.1), as it was similarly observed for MOIs 1 and 10 (Fig S4). Together, these results clearly demonstrate that SLC3A2 modulates ExoU-dependent cytotoxicity.

To further examine the intracellular colocalization between the two proteins, ExoU and SLC3A2 were labeled in A549 WT cells and WT murine fibroblasts infected with an ExoU^{S142A} expressing strain in a Δ*exoSexoT* background. Images at the top, middle and bottom of the cells were acquired. Analysis reveal a partial colocalization between ExoU and SLC3A2 in both cell lines (Fig 6C). This colocalization also confirms the PL data indicating that ExoU and SLC3A2 localize in close proximity during host cell infection.

Association with host exocytic proteins alleviates the intracellular burden of ExoU

To assess the role of SNAP23 in ExoU activity, SNAP23 KO HEK cells were obtained from D. Michiel Pegtel lab, Amsterdam (54). These cells express a hemagglutinin (HA)-tagged CD63 fused to nanoluciferase « CD63-HA-NL » (54). CD63 is a tetraspanin and a marker of extracellular vesicles (EVs) shaping their biogenesis (55,56). SNAP23 drives the docking and the fusion of multivesicular bodies (MVBs) with the PM leading to the release of EVs (57,58) thus being in the same pathway as CD63. In a similar set of experiments as for SLC3A2 fibroblasts, we found that SNAP23 KO cells presented a significantly higher AUC in cytotoxicity experiments, showing that they were more sensitive to ExoU than the parental WT cells (Fig 7A). Whereas both cell lines were similarly sensitive to the ExlA (IHMA87) and ExoS activities

as no significant difference was observed between the respective normalized AUCs (Fig 7A and B). We also infected both cell lines with increasing MOIs using the Δ3tox-ExoU strain and obtained a significant increase in the cytotoxicity for all MOIs (Fig S5), highlighting again that the sensitive phenotype is MOI-independent. As we were unsuccessful to specifically label endogenous SNAP23 using available α-SNAP23 antibodies, we performed a colabeling of ExoU and CD63. Both proteins were labeled in HEK WT cells infected with an ExoU^{S142A} expressing strain in a Δ*exoSexoT* background. Image analysis shows a colocalization between ExoU and CD63, occasionally within vesicle-like structures (Fig 7C).

ExoU is detected in extracellular vesicles

During eukaryotic host infection, bacteria interact with the hosts' trafficking pathways; resulting either in pathogen elimination or the subversion of these pathways by the pathogen (59,60). Since the SNAP23 KO cells were more sensitive to ExoU, we hypothesized that SNAP23/CD63 could be part of a detoxification pathway to eliminate cytotoxic ExoU. Therefore, we wondered whether ExoU can be found in EVs produced by eukaryotic cells infected with *Pa*. We infected HEK WT cells with *Pa*-ExoU^{S142A} and proceeded to isolate EVs from supernatants. Using established protocols (61), the pellet after centrifugation at 100 000g for 1 hour was analyzed by Western blot. We included the EVs from non-infected and infected cells supernatants in addition to *Pa*-ExoU^{S142A} bacterial lysate. The immunoblot revealed with anti-CD63, shows the presence of CD63 in both EVs samples (Fig 7D). Probing the membrane with anti-RpoA, a marker of bacterial cytosol, showed that there was no contamination with bacterial proteins in the EVs of the infected samples (Fig 7E). Finally, ExoU was detected in the EVs of the infected cells supernatants (Fig 7F), showing that ExoU is present in EVs originating from infected HEK cells. This supports the hypothesis that ExoU is detoxified *via* an exocytic pathway, mediated by SNAP23 and CD63.

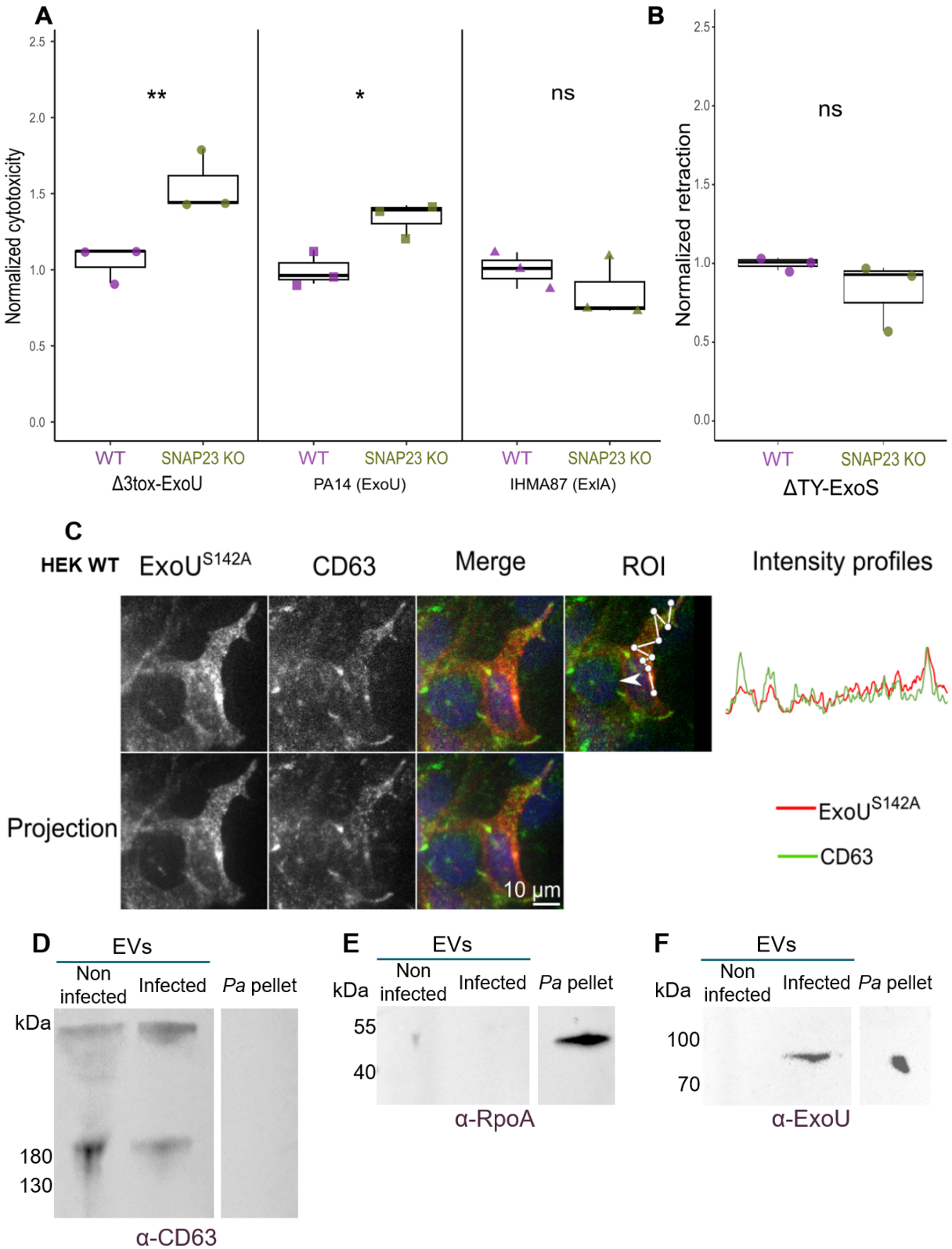


Fig 7. A SNAP23 dependent pathway detoxifies ExoU through EVs. A. WT or SNAP23 KO HEK cells infected with *Pa* strains Δ3tox-ExoU, PA14 or IHMA (MOI 0.1). Cell death was monitored by live-cell imaging and quantified by PI-positive cells over time. Data were normalized and the area under the curves (AUC) was calculated for each condition. WT cells are shown in purple, SNAP23 KO cells in green and individual values are represented as dots. **B**. WT or SNAP23 KO HEK cells infected with ΔTY-ExoS *Pa* strain (MOI 0.1). Cell retraction was monitored by live-cell imaging and quantified by the number of retracted cells over time. Data were normalized and the area under the curve (AUC) was

calculated for each condition. **: $p < 0.01$, *: $p < 0.05$, ns: not significant $p \geq 0.05$. **C.** HEK WT cells were infected for 4 hours with *Pa*-ExoU^{S142A} and stained for CD63 (green, shown in grayscale) and ExoU (red, shown in grayscale). A single Z-plane is shown and a Z-projection. Columns display from left to right: SLC3A2 staining (grayscale), ExoU staining (grayscale), merge, merge with a manually selected region of interest (ROI) used for quantification and intensity profiles. **D, E and F**. Western blots of EVs isolated from non infected HEK WT HA-CD63-NL cells or infected with CHA-ExoU^{S142A} *Pa* strain cells and a lysate of bacterial pellet (*Pa* pellet). Membranes were revealed with: α-CD63 as marker of EVs (**D**), α-RpoA as a marker of bacterial cytosol (**E**) and α-ExoU (**F**) antibodies.

Discussion

The aim of this study was to get further insights on the ExoU host proxisome during infection using PL. For the first time, we successfully applied PL on a T3SS injected effector of the extracellular pathogen *P. aeruginosa*. PL identified novel host proteins in the close environment of *P. aeruginosa*'s potent toxin ExoU during infection. This was followed by a targeted functional characterization of two proteins, SLC3A2 and SNAP23, which revealed their protective roles against ExoU-mediated cytotoxicity rendering host cells more susceptible in their absence.

For all strains and constructs tested, the fusion proteins were less injected compared to ExoU alone. This difference could be due to the less efficient handling of the fusion proteins by the T3SS machinery. Precisely, effectors are recruited, sorted and properly unfolded by dedicated ATPases to be able to travel through the T3SS needle (62). In addition, bulky and tightly folded proteins can obstruct the needle and affect the overall T3SS toxins secretion in a given *Pa* strain (63,64).

In our hands, several biotinylation attempts failed to detect the enriched auto-biotinylated ExoS, ExoT and ExoY fusions despite their injection in contrast to what we observed for the ExoU fusion. These fusions could be either cleaved while in the bacteria (65,66) or proteolysed in the host cytoplasm (67). This could also be linked to differences in the intracellular localization of ExoU compared to the other toxins, potentially affecting biotin access (68). Alternatively, the fusion protein might be unable to refold properly or folds in a way that masks the active site of UltraID (69,70)

Previous works identified the membranous PI(4,5)P2 as a cofactor directly interacting with ExoU (19) and inducing conformational changes within ExoU leading to its PLA2 activity (71). Bagayoko et al., reported that ExoU PLA2 activity is enhanced by host peroxidised phospholipids eventually leading to cell death by necrosis where it was suggested that ExoU takes advantage of host ferroptosis pathways (72). Ferroptosis is a subtype of unbalanced lipid peroxidation-induced cell death (73). It is known that SLC3A2 takes part in the regulation machinery of ferroptosis (74). SLC3A2 is a subunit of the heterodimer that imports cysteine that is necessary for the synthesis of glutathione (GSH). Then glutathione peroxidase 4 (GPX4) uses GSH to reduce peroxidised lipids (75). Therefore, when SLC3A2 is absent, the whole downstream cascade is affected; GSH is missing, GPX4 is inactivated and peroxidised lipids are accumulated strongly enhancing ExoU-induced cell death. In accordance with this, murine dermal fibroblasts lacking SLC3A2 presented a higher sensitivity to ExoU necrotic activity. It is worth mentioning that SLC3A2 is enriched in lipid rafts (76) and ExoU is targeted to its PM cofactor, the PI(4,5)P2, also concentrated in lipid rafts (77).

In addition to PI(4,5)P2, ubiquitin is also required for ExoU activation as it interacts with its C-terminal domain (aa 588 to 687) inducing conformational changes critical for the PLA2 activity of ExoU (71,78,79). ExoU is ubiquitinated at the lysine 178 residue, a modification proven not to be required for its localization to DNAJC5 vesicles (25) nor for its phospholipase activity (20,78). Thus, it would be interesting to investigate whether this ubiquitination affects the colocalization of ExoU with SLC3A2 and CD63 in addition to its association with EVs. Furthermore, the C-terminus of ExoU, which contains the membrane localization domain, can be tested for its potential role in the targeting of ExoU to regions harboring the identified host factors.

ExoU uses DNAJC5 vesicles to reach the PM, which makes DNAJC5 indispensable for ExoU-induced cell death (25). Based on our observations, ExoU is either targeted to the PM through DNAJC5 vesicles therefore causing cell death or it is recognized and directed for elimination, possibly through several host defense mechanisms. SLC3A2 is involved with MAPS and more

specifically it is required for the perinuclear association between MAPS and DNAJC5 (48). In DNAJC5$^{-/-}$ cells, ExoU is found in membranous fractions (25) and in our hands, the enrichment of SLC3A2 enrichment was exclusive in A549 WT compared to the DNAJC5$^{-/-}$. This suggests that DNAJC5 vesicles target ExoU to specific regions at the PM where SLC3A2 is present. Alternatively, DNAJC5 vesicles can produce EVs (80), hence, we could be looking at a dual role for DNAJC5 in the ExoU journey, the detoxification by DANJC5 EVs being of minor effect on the cytotoxicity of ExoU.

In addition, SLC3A2 is recognized as a marker of PM-derived EVs (ectosomes) found on CD63 EVs (81). Further analysis should focus on the identification of the possible different EVs populations in addition to their proteins content. As this could be one possibility for ExoU to reach the EVs.

Lastly, after the localization with early endosome antigen 1 (EEA1) (20), ExoU might traffic through the endosomal pathway reaching MVBs marked with CD63 and is then released within EVs in a SNAP23-dependent pathway. Based on these various possibilities, ExoU is indeed spatio-temporally distributed in a heterogeneous manner across different compartments within host cell during infection. Therefore, future studies should be dedicated to identifying and characterizing these compartments, as well as deciphering the molecular mechanisms governing the association of ExoU.

The KO and their parental cell lines were similarly susceptible ExoS and ExlA, other T3SS and PM-damaging toxins respectively. Thereby highlighting that SLC3A2 and SNAP23 KOs display an ExoU-exclusive sensitive phenotype. This observation could be associated to the different localization, activation and mode of action of each toxin.

Several ExoU homologs with similar domains have been identified and predicted (82). Therefore, investigating the roles of SLC3A2 and SNAP23 in mediating their cytotoxic effects would be of particular interest.

In conclusion, this study provided insights into the host cell response toward ExoU cytotoxicity. We identified host factors with a protective role against the infection with an ExoU^{+} *Pa* strain. Despite these mechanisms, the cytotoxicity of ExoU overwhelms the eukaryotic cells and the associated cellular defenses eventually leading to cell death. Stimulation of detoxifying pathways could be of advantageous in combating infection with *Pa* ExoU^{+} strains.

Material and methods

Bacterial strains, constructions, and genetic manipulations

Bacterial strains, plasmids, and primers are listed in supplementary tables 1 and 2 (ST1 and ST2). Plasmids were cloned and prepared in *E. coli* Top10 competent cells and selected on LB plates containing tetracycline 10 µg/mL or ampicillin 100 µg/mL for the pminiCTX and pUCP20 plasmids respectively. UltraID, BioID2 and iFAST fragments were amplified with Q5 High-Fidelity DNA polymerase from their respective addgene plasmid using appropriate primer pairs. Overlapping fragments were cloned into *Hind*III *a*nd *Xba*I-digested pUCP20 ExoU^{S142A}-bla (to excise the bla fragment) by Sequence- and Ligation-Independent Cloning (SLIC, (83). ExoU^{S142A} is herein referred to as ExoU* (inactive form). Plasmids were transformed into Top10 cells and their sequences were verified by DNA sequencing (Eurofins). These constructs were then digested by *Hind*III and *BamH*I, cloned into similarly digested miniCTX plasmid. The miniCTX plasmids were introduced into *P. aeruginosa* by triparental mating, using pRK600 as a helper plasmid. *P. aeruginosa* was selected on LB plates containing 10 µg/mL irgasan and 75 µg/mL tetracycline. The same strategy was applied to obtain the WT ExoU (active form) fusions but with the starting backbone as pUCP20 WT ExoU-Bla.

To obtain the pUCP20 ExoU*-UltraID SpcU plasmid, the UltraID fragment was amplified with a different pair of primers. Overlapping fragments were cloned into *Spe*I-digested pUCP20 ExoU*-BioID SpcU (to excise BioID) by SLIC, therefore UltraID was cloned between ExoU* and SpcU. To obtain the catalitically active construct, the UltraID-SpcU fragment was amplified from the pUCP20 ExoU*-UltraID SpcU with appropriate primers. Overlapping fragments were

cloned, by SLIC, into the *Hind*III and *Xba*I digested pUCP20 ExoU-iFAST (to excise iFAST). The pUCP20 plasmids were introduced into *P. aeruginosa* by transformation. *P. aeruginosa* was selected on *Pseudomonas* isolation agar (PIA) plates containing 300 µg/mL carbenicillin.

Complete sequences could be provided upon request.

Bacterial growth conditions

All strains were grown at 37 °C in Lysogeny Broth (LB) under constant shaking at 300 rpm. *P. aeruginosa* strains with the pUCP20 plasmid were grown overnight in the presence of 300 µg/mL carbenicillin. Unless stated otherwise, overnight cultures were diluted in fresh LB medium to an initial optical density at 600 nm (OD_{600}) of 0.1 and left to grow to the desired OD_{600}. *E. coli* strains were grown in the presence of 10 µg/mL tetracycline or 100 µg/mL ampicillin to maintain the pminiCTX or pUCP20 plasmids respectively.

Eukaryotic cell lines and culture conditions

All cell lines were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. A549 (ATCC CCL-185) and DNAJC5$^{-/-}$ (25) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Murine wild-type (WT) and SLC3A2 knockout (KO) dermal fibroblasts were kindly provided by Dr. Chloé Féral (INSERM U1081, CNRS UMR7284, Université Côte d'Azur, IRCAN, Nice, France). Both cell lines were maintained in a custom culture medium consisting of DMEM, 10% FBS, 1× MEM non-essential amino acids, 20 mM HEPES, and 0.1 mM β-mercaptoethanol.

Human embryonic kidney HEK 293-5610 HA-NL-CD63 WT and its isogenic SNAP23 KO derivative were obtained from the laboratory of Dr. Michiel Pegtel (Department of Pathology, Cancer Center Amsterdam, VU University Medical Center, Amsterdam, Netherlands). These cells stably express a CD63–NanoLuciferase fusion protein to monitor the secretion of exosomes/EVs. Both cell lines were cultured in DMEM supplemented with 10% FBS and 1×

penicillin/streptomycin (P/S). For all cultures, the FBS was heat-inactivated at 56°C for 30 minutes.

siRNA transfection

Experiments were performed as previously described (25). A549 WT cells were seeded at 3 × 10^5 cells per well in a 6-well plate (cell lysis and WB analysis) or at 1.25 × 10^4 cells per well in a 96-well plate (infection assay) in DMEM 10% FBS 24 h before transfection. Transfection was carried out using the Lipofectamine RNAiMax kit according to the manufacturer's instructions (one or two siRNAs per target) with 3 pmols siRNAs per well of 96-well plate or 30 pmols per well of 6-well plates. After 24 h of transfection, the media were replaced with fresh DMEM 10% FBS. Cells in the 6-well plates were lysed at 1, 2 or 3 days post-transfection to monitor the downregulation of the respective proteins (lysis buffer : 1% triton x-100, 50 mM tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and protease inhibitor cocktail). The protein's content in the lysate was quantified using the Micro-BCA Protein Assay Kit and similar quantities were loaded onto the SDS-gel to be analysed by WB. Cells were infected with the PA14 strain at MOI 20 48 h post-transfection. This experiment was performed two times each with 6 technical replicates per condition.

***In vitro* secretion assay of the T3SS**

Overnight cultures were diluted to an OD_{600} of 0.1 and grown until OD_{600} 0.8-1.2 in the presence or not of 5 mM Ethylene Glycol Tetraacetic Acid (EGTA) and 20 mM $MgCl_2$. Similar amounts of bacteria were taken from each condition, centrifuged at 8000 rpm for 10 minutes to separate the pellet (intracellular) from the supernatant (secretion). Samples were then denatured using Laemmli buffer containing Dithiothreitol (DTT) at 98°C for 10 minutes and then analyzed by western blot (WB). Similar volumes were loaded of the pellets and supernatants of each condition.

Western blot

Samples were loaded on 12% SDS-PAGE, 4-12% Bis-Tris or 4-20% Tris-Glycine gels. Western blotting analyses were done on polyvinylidene difluoride (PVDF) 0.22µm transfer membranes after electrotransfer in buffer containing 20% ethanol and 10% TG-SDS. The membranes were blocked with 5% milk in Phosphate Buffer Saline (PBS) + Tween 0.1% (v/v) overnight. Washes with PBS Tween 0.1% were performed before and after the incubation with primary antibodies followed by secondary horseradish peroxidase (HRP)-conjugated antibodies. Biotinylated proteins were revealed with Streptavidin-HRP. All antibodies were diluted in 1% milk PBS Tween 0.1%. Detection was performed with Immobilon Crescendo Western HRP substrate.

Cytotoxicity assays

A549 cells. WT and DNAJC5$^{-/-}$ cells were seeded at 50 000 cells per well 1 day before infection and bacterial cultures were launched. Before infection, the medium was replaced with DMEM 10 % FBS containing 1 µg/mL propidium iodide (PI). When bacteria cultures reached OD_{600} = 0.8 – 1.2, bacteria were added to the cells at a multiplicity of infection (MOI) of 10 for all strains except the ExoU-BioID2 and ExoU-UltraID expressing strains (MOI 100). Data was acquired, as incorporated PI red fluorescence signal, on a Fluoroskan microplate reader (Thermo). Each experiment was performed in triplicates. When mentioned, the PI fluorescence of triplicate was normalized to the PI fluorescence obtained with the parental *Pa* strain. Data was processed in R Studio to calculate the Area Under the Curve (AUC) as previously described by (84).

Fibroblasts WT, SLC3A2 KO, HEK 293 T WT and SNAP23 KO cells. Cytotoxicity assays were performed using the IncuCyte® ZOOM live-cell imaging system (Sartorius). Murine dermal fibroblasts (5,000 cells/well) and HEK 293-derived cells (10,000 cells/well) were seeded 24 hours prior to infection. For HEK 293 cells, wells were coated with poly-L-lysine 0.001% before seeding. One hour before infection, cells were incubated with SYTO24 to label

nuclei, then washed and incubated with DMEM supplemented with 10% FBS and PI. Infections were carried out at a multiplicity of infection (MOI) of 0.1 unless stated otherwise.

Phase-contrast and red channel images were acquired every 15 minutes for 16 hours using a 10× objective. The SYTO24 signal was (green channel) used to assess initial cell density at the first time point and verify comparability between conditions but was not included in the final cytotoxicity analysis. Quantification of cell death was performed automatically using the IncuCyte software, based on PI fluorescence intensity (PI-positive cell count). All experimental conditions were monitored in technical triplicates. Cytotoxicity assays were performed in standard 96-well Falcon® microclear plates (Corning). Plates for the Fluoroksan (353072) and for the IncuCyte (353219) experiments. All cytotoxicity assays were performed 3 to 5 times each with 3 technical replicates.

Image analysis and quantification of the Incucyte data

Cell death. Time-lapse images were analyzed using the IncuCyte software to quantify cell death, based on the number of PI-positive nuclei per image. For each image, the PI signal was normalized by dividing the number of PI-positive cells at each time point by the maximum value reached in that well at 100% of cell death at the end of infection.

Normalized curves were used to compute the area under the curve (AUC) using a fixed time interval, defined as the time point at which WT cells reached maximal PI incorporation. AUCs were then normalized by the mean AUC of the WT condition for each bacterial strain, to allow inter-condition comparison across experiments. Boxplots were generated with RStudio to visualize normalized AUCs by bacterial strain.

Cell retraction. Phase-contrast images were exported from the IncuCyte system as uncompressed .avi files and analyzed using Fiji software. Stacks were opened as virtual stacks, converted to grayscale and concatenated by well (A10–H12). Image stacks were cropped and filtered using a Gaussian blur ($\sigma = 2$) to reduce background noise generated by bacteria adhering to the plate during infection. To detect retraction-associated changes, the

"Find Maxima" function was applied to each frame with a fixed noise tolerance, generating time-resolved counts of contrast maxima. These maxima reflect the appearance of rounded, highly contrasted cells during retraction. Resulting counts were exported to Excel and further processed in RStudio. Curves were normalized as described above for cell death analysis. For HEK cells, data were additionally background-corrected by subtracting the lowest value in each condition, to set all curves to a baseline of zero. Normalized AUCs were used for inter-condition comparison, and statistical analysis was performed as described below.

Proximity labeling assay – biotinylation

Plates containing 20 x 10^6 A549 WT or DNAJC5$^{-/-}$ cells were used during this experiment (in triplicates). Cells were infected with the overnight cultures of each strain respectively at a MOI 100. Biotin was added (or not) at 500µM at 2.5 h post-infection. At 6 h post-infection, cells were washed 3 times with PBS. A new medium supplemented with 40 µg/mL PolymixinB, 300 µg/mL Gentamicin and 500 µM biotin (or not, only in Fig. 2) was added to the plates to stop the infection and kill the bacteria. Two hours later, the cells were washed, harvested, centrifuged and lysed in: 50 mM Tris–HCl, 500 mM NaCl, 5 mM, EDTA, 0.4% SDS, 1 mM DTT and protease inhibitor cocktail, pH adjusted to 7.4. In the first biotinylation test, we biotinylated for one hour during a 6 hours infection with the ExoU*-UltraID strain and 18 hours during a 21 hours infection with the ExoU*-BioID2 strain (Fig. 1). The lysates (input) were incubated overnight with streptavidin magnetic beads (Dynabeads MyOne Streptavidin C1) at 4°C. The beads were succesively washed with: lysis buffer, 1 M KCl, 100 mM Na_2CO_3, 2 M urea in 10 mM tris HCl pH 8 and lysis buffer. The beads were stored dry at -80°C to be analysed by MS or eluted (output) in Laemmli buffer containing DTT and heated at 98°C for 15 minutes to be analysed by WB. All biotinylation assays for the MS analyses were performed in triplicates.

Mass spectrometry analyses

Proteins were digested on beads with trypsin (sequencing grade modified trypsin, Promega) as described in (85). The resulting peptides were desalted on SPE tips (BioSPE PurePep, Affinisep) before being analysed by nanoliquid chromatography (Ultimate 3000 RSLCnano,

Thermo Fisher Scientific) coupled to a mass spectrometer (Q Exactive HF or Orbitrap Exploris 480, Thermo Fisher Scientific). The mobile phases were composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.08% formic acid (solvent B); a 90 minutes gradient was used, with an increase of solvent B, from 2% to 72.2%. Peptides were loaded on a precolumn (PepMap C18, 300µm x 5mm, Thermo Fisher Scientific) and then separated on a reverse-phase column (Aurora Ultimate 25cm x 75µm C18, IonOpticks). MS2 data were acquired using a data-dependent acquisition strategy.

Peptides and proteins were identified by Mascot (version 2.8.0, Matrix Science) using the Uniprot database (*Homo sapiens* and *Pseudomonas aeruginosa - strain UCBPP-PA14* taxonomies), and homemade databases containing the sequences of classical contaminants and of tagged proteins. For the search parameters, trypsin was set as the enzyme with 2 missed cleavages allowed, the cysteine carbamidomethylation was set as a fixed modification while the N-term acetylation and the methionine oxidation were set as variable modifications. Peptide mass tolerance was set at ± 10 ppm and fragment mass tolerance at ± 20 ppm.

Filtering and validation of the data were made using Proline Software ((86), version 2.3.0). The validation parameters were set as followed: conservation of rank 1 peptides, peptide length ≥ 6 amino acids, false discovery rate of peptide-spectrum-match identifications < 1% (87), minimum of one specific peptide per identified protein group. Proline was then used to perform a MS1 label-free quantification of the identified protein groups based on razor and specific peptides.

Statistical analysis of quantitative proteomic data was performed using the Prostar software ((88), version 1.30). Proteins identified in the contaminant database, proteins identified in less than two replicates of one condition and proteins quantified in less than three replicates of one condition were discarded. After log2 transformation, abundance values were normalized by Variance Stabilizing Normalization (vsn) method or normalized on ExoU. Statistical testing for comparison of two conditions was conducted with limma, whereby differentially expressed proteins were sorted out using a log2(Fold Change) cut-off of 1 and a p-value cut-off of 0.01,

leading to a FDR inferior to 3 % for each comparison according to the Benjamini-Hochberg estimator.

Fluorescence microscopy labeling and analyses

Fibroblasts (25 000 cells/well), HEK (50 000 cells/well) or A549 (50 000 cells/well) were seeded in Lab-Tek 8-well chamber slides 24 h prior to infection. Cells were infected with the CHA-ExoU^{S142A} for 4 h at OD 1 (MOI 10). After infection, cells were fixed overnight at 4 °C with 4% paraformaldehyde (PFA) and permeabilized for 5 minutes with 0.5% Triton X-100 in PBS. Samples were blocked with 2% bovine serum albumin (BSA) for 30 minutes before staining with primary and secondary antibodies. The following primary antibodies were used: anti-ExoU (1:1000, gift from S. Barzu, Sanofi Pasteur), anti-CD63 (1:1000, BD Pharmingen), anti-SLC3A2 human (1:1000, Proteintech), anti-SLC3A2 murine (1:1000, Absolute Antibodies), and anti-SNAP23 (1:500, Invitrogen). Nuclei were counterstained with Hoechst 33258 (Sigma-Aldrich). Images were acquired on a spinning disk confocal microscope (Olympus IX81, GATACA systems, Andor iXon EMCCD camera) equipped with a 60× oil immersion objective (NA 1.4). Z-stacks were collected every 0.2 µm. Images of z-sections were analyzed using the Intensity Profile module from Icy software. This experiment was performed one time.

Extracellular vesicles isolation

Four plates (145 cm^2) of 20 x 10^6 HEK 293 T WT cells each were infected or not by CHA-ExoU^{A142} strain at OD 1 (MOI 10). Six hours later, the supernatants (15 mL per plate) of each condition were collected, pooled together and supplemented with protease inhibitor cocktail. A first centrifugation step at 4000 rpm for 15 minutes was performed followed by two 30 minutes 20 000 g (Ti 45 rotor) centrifugations at 4°C. The supernatants were then filtered with a 0.22 µM membrane low protein binding filter to eliminate all bacteria. The pellet after a last 1 h centrifugation at 100 000 g (Ti 45 rotor) at 4°C was resuspended in 200 µL of PBS and was analyzed by Western blot. This experiment was performed two times.

Chemicals, reagents, antibodies and siRNAs

Name	Reference	Provider
Reagents & materials		
HEPES	11344-041	Gibco BRL
Phosphate Buffer Saline (PBS)	14190-064	Gibco
Trizma base	T1503-1KG	Sigma-Aldrich
Dithiothreitol (DTT)	D0632-10G	Sigma-Aldrich
ß-mercaptoethanol	M3148-250ML	Sigma-Aldrich
SDS 20%	EU0660-B	Euromedex
Tween-20	P1379-250ML	Sigma-Aldrich
Triton x-100	T8787-100ML	Sigma-Aldrich
NaCl	71383-1KG	Sigma-Aldrich
$MgCl_2$ 1M	M1028-100ML	Sigma-Aldrich
KCl	7447-40-7	Carlo Erba Reagenti
Na_2CO_3	27 771.290	Prolabo
Urea	1.08488.1000	Merck
EDTA	E5134-1KG	Sigma-Aldrich
EGTA	E4378-100G	Sigma-Aldrich
Ammonium hydroxide	221228-500mL	PCA
Biotin	B4501-1G	Sigma-Aldrich
Propidium iodide (PI)	P3566	Invitrogen
Protease inhibitor cocktail	237072	Roche
One Tag Hot Start Quick-Load Master Mix	M0489S	New England BioLabs
Q5 High-Fidelity DNA polymerase	M0491L	New England BioLabs
Dynabeads MyOne Streptavidin C1	65001	Invitrogen
Polyvinylidene difluoride (PVDF) membrane	10600021	Cytiva
Immobilon Crescendo Western HRP substrate	WBLUR0100	Millipore
Lipofectamine RNAiMax kit	13778075	Invitrogen
Micro-BCA protein assay kit	23235	Thermofisher
Paraformaldehyde (PFA) 16%	15710-S	Electron Microscopy Sciences
Streptavidin-HRP	7403	Abcam

Poly-L-lysine	P4107-50ML	Sigma-Aldrich	
Nanoluc kit			
Mediums and antibiotics			
Lysogeny Broth (LB)	244610	Becton Dickinson	
Pseudomonas Isolation Agar (PIA)	17208-500G	Millipore	
Dulbecco's modified Eagle's medium (DMEM) - GlutaMAX	31966-047	Gibco	
Fetal bovine serum (FBS)	10270	Gibco	
Non-essential amino-acids	11140-035	Life Technologies	
Carbenicillin	C1389-5G	Sigma-Aldrich	
Ampicillin	A9393-5G	Sigma-Aldrich	
Tetracyclin	T7660-25G	Sigma-Aldrich	
Irgasan	72779-25G-F	Sigma-Aldrich	
PolymixinB	P4932-5MU	Sigma-Aldrich	
Gentamicin	EU0410-B	Euromedex	
Penicillin-Streptomycin	15140-122	Life Technologies	
siRNAs			
SLC3A2	s12944 s12943	ThermoFisher Scientific	
SNAP23	s16709 s16710	ThermoFisher Scientific	
RAB27B	s224511	ThermoFisher Scientific	
DNAJC5	s37237 s37238	ThermoFisher Scientific	
Non-targeting	D-001210-01-20	Dharmacon	
Antibodies			**Dilution**
ExoU	(25)	Gift from S. Barzu, Sanofi-Pasteur	1 : 5000 WB 1 : 1000 IF
β-actin	A1978	Sigma-Aldrich	1 : 5000 WB
rpoA	663104	Biolegend	1 : 5000 WB
CD63	556019	BD Pharmingen	1 : 1000 WB
RAB27B	13412-1-AP	Proteintech	1 : 2000 WB
SNAP23	PA1-738	Invitrogen	1 : 3000 WB 1 : 1000 IF
Human SLC3A2	66883-1-Ig	Proteintech	1 : 5000 WB 1 : 1000 IF
Murine SLC3A2	Ab01268-7.1	Absolute antibody	1 : 1000 IF

Anti-rabbit HRP	A9169	Sigma	1 : 25000 WB
Anti-mouse HRP	A9044-2ML	Sigma	1 : 25000 WB
Anti-rabbit A488	A21206	Molecular Probes	1 : 500 IF
Anti-mouse Cy3	715-165-151	Jackson ImmunoResearch Laboratories	1 : 500 IF
Hoescht 33258 (Probes)	861405	Sigma Aldrich	1 : 1000 IF

WB: Western blot, IF: immunofluorescence, HRP: horseradish peroxidase

Statistical analyses

Statistical analyses were performed using R (v. 4.4.2; R Core Team, 2024) within RStudio (89). Normality of data distribution was assessed using Shapiro–Wilk's test, and equality of variances using Levene's test. When assumptions of normality and homoscedasticity were met, comparisons between two groups were performed using Student's t-test. For multi-condition analyses, two-way ANOVA was applied, followed by pairwise t-tests with Benjamini–Hochberg correction for multiple comparisons. Significance levels are indicated as follows: $p < 0.05$ (), $p < 0.01$ (), $p < 0.001$ (), and n.s. indicates a non-significant difference.

Acknowledgments

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Supplementary figures and legends

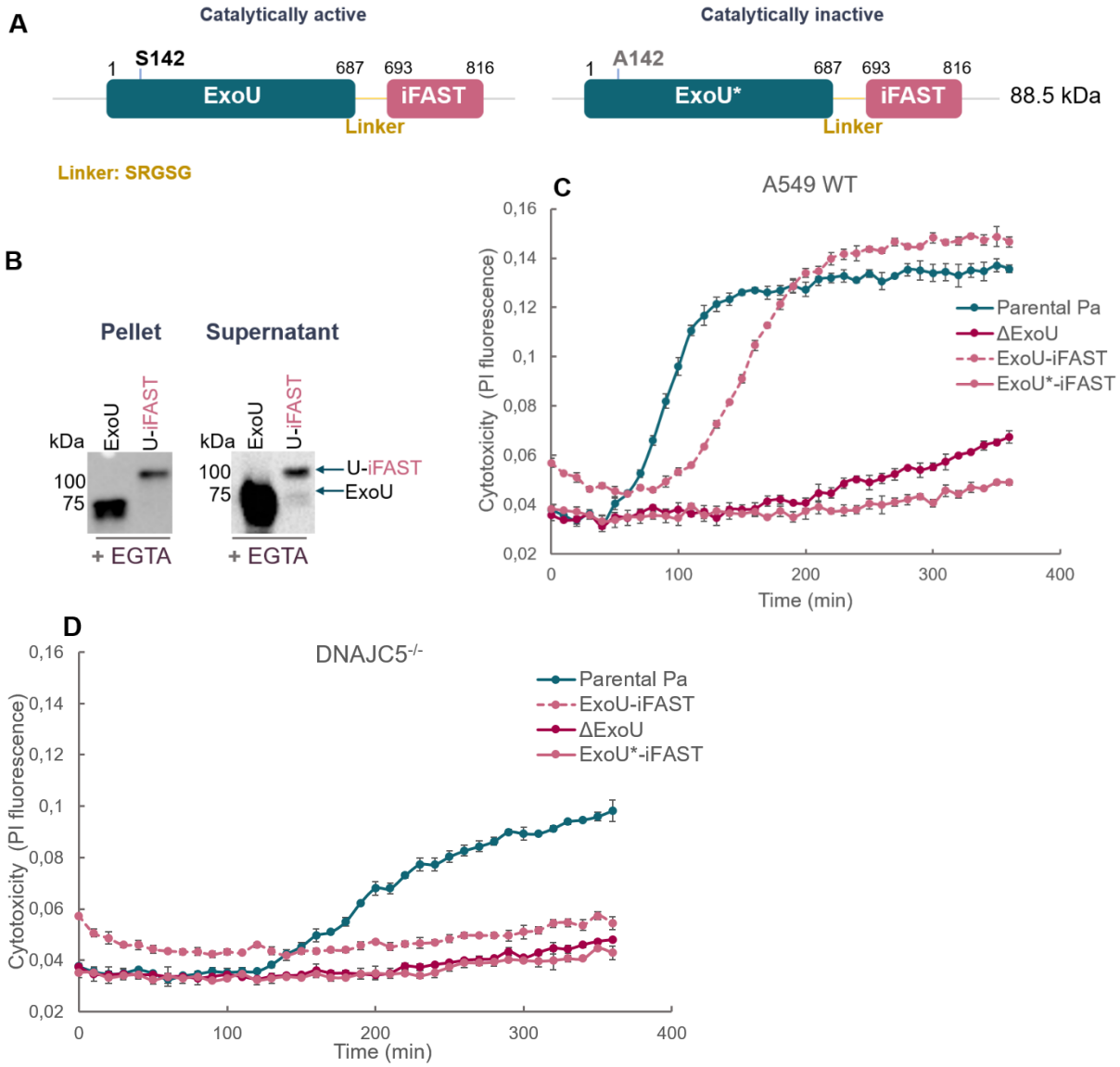

Fig S1. Engineered ExoU-iFAST is secreted, active and requires DNAJC5. A. Schematic representation of the ExoU-iFAST fusion proteins constructs inserted into the genome of a Δ*exoU* strain. Active ExoU (S142) and inactive ExoU* (A142) fusions were generated. A linker (SRGSG) separates ExoU and iFAST. **B.** Western blot showing the expression of ExoU-iFAST and its secretion in the supernatant upon an *in vitro* induction (+EGTA) of the T3SS compared to the unmodified ExoU. Western blot revealed with α-ExoU antibody **C and D.** Cell death of A549 WT (**C**) and DNAJC5$^{-/-}$ (**D**) cells upon infection with ExoU-iFAST expressing strains compared to the parental and ΔExoU *Pa*. Cytotoxicity is represented as mean ± standard deviation (SD) of PI fluorescence signal over time.

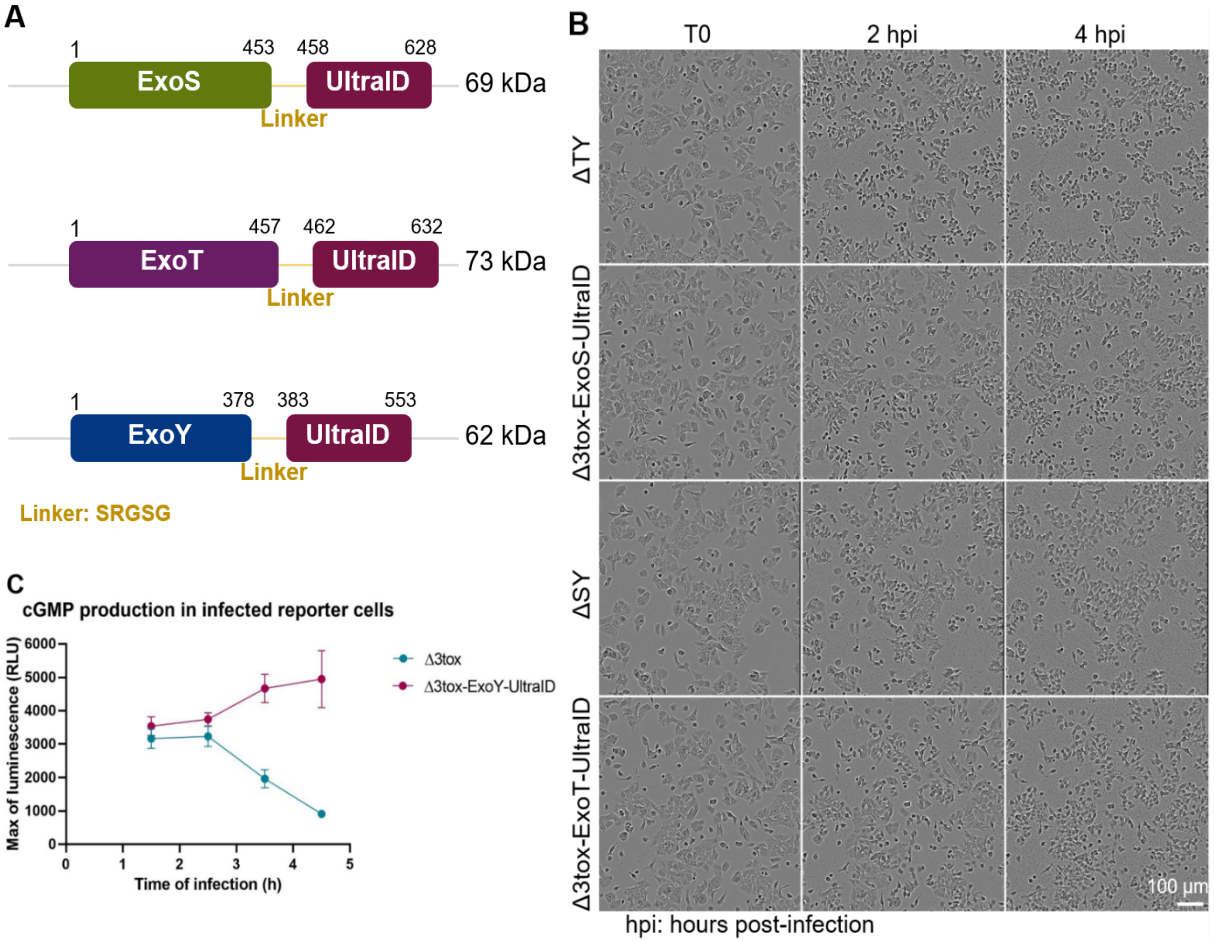

Fig S2. Engineering and activity of ExoS, ExoT and ExoY fused to UltraID. A. Schematic representation of the ExoS-UltraID, ExoT-UltraID and ExoY-UltraID fusion proteins constructs inserted into the genome of a Δ*exoU* strain. A linker (SRGSG) separates the toxin from UltraID. **B.** Comparison of ExoS and ExoT fusion toxins activity assays by monitoring host cells. A549 WT cells were infected with the Δ3tox strain expressing either the ExoS-UltraID or ExoT-UltraID fusion compared to ΔTY and ΔSY strains respectively. Infection was monitored by live-cell imaging at different stages of infection: transmitted light images of the same field were acquired at 0, 2, and 4 hours post-infection. **C.** ExoY-UltraID fusion activity assay by tracking host intracellular cGMP levels during infection compared to Δ3tox background strain. Luminescences are presented as mean value ± standard deviation (SD).

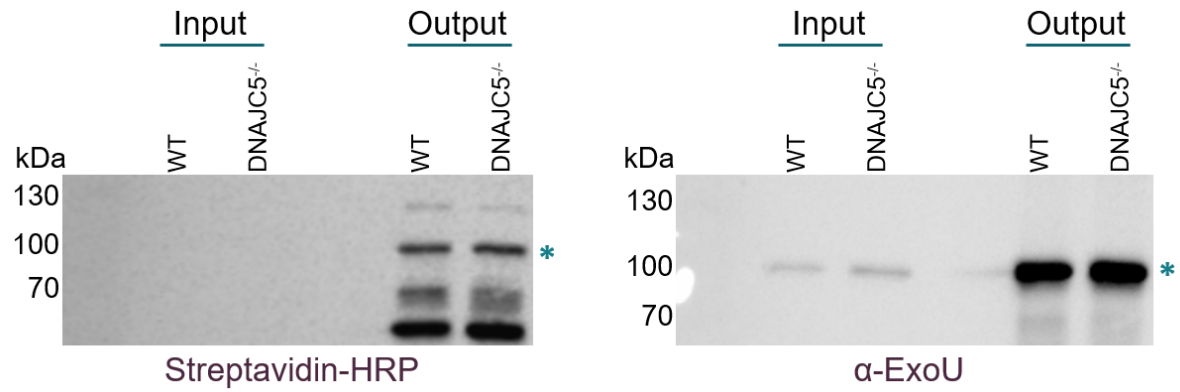

Fig S3. Biotinylation test validates the use of the DNAJC5$^{-/-}$ cells and the newly constructed PAO1Δ3tox//ExoU*-UltraID strain for PLA. Western blot revealed with streptavidin-HRP and with α-ExoU showing the enrichment of biotinylated proteins after PLA (input *vs* output). A549 WT and DNAJC5$^{-/-}$ cells were infected with PAO1Δ3tox//ExoU*-UltraID strain and biotin was added + at 500 µM for 5.5 hours during infection. Auto-biotinylated ExoU*-UltraID ~100 kDa is indicated by an *.

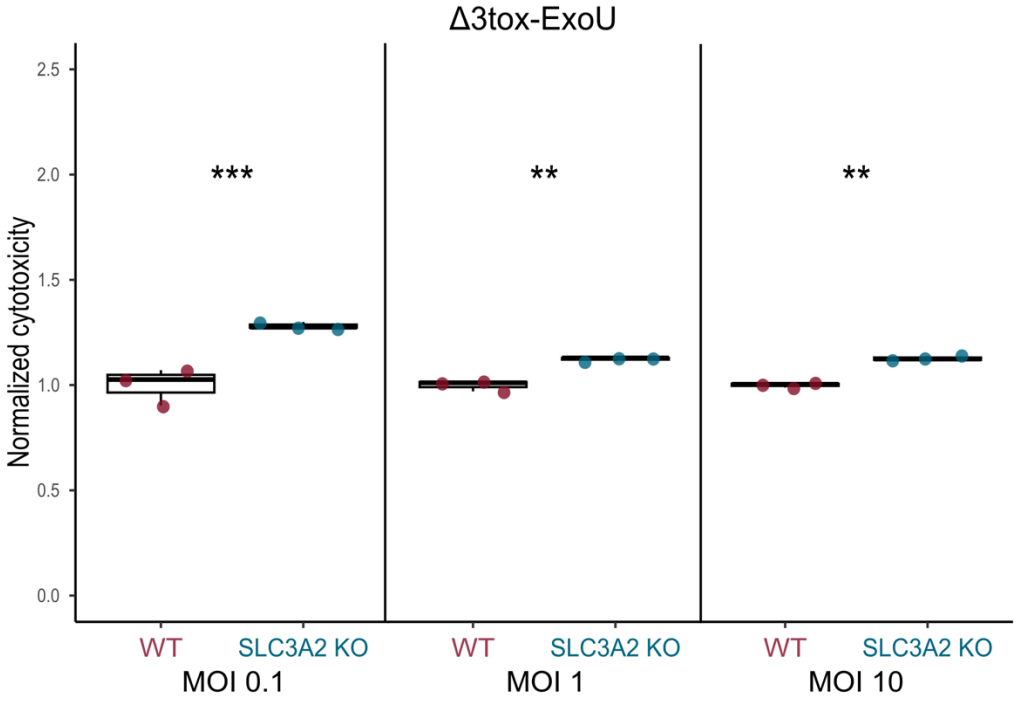

Fig S4. The SLC3A2 KO sensitivity phenotype is independent of the MOI. WT or SLC3A2 KO dermal murine fibroblasts infected with Δ3tox-ExoU *Pa* strain at increasing MOIs (0.1, 1 and 10). Cell death was monitored by live-cell imaging and data was normalized. The AUC of each condition is presented. WT cells are shown in red, KO cells in blue and individual values are represented as dots. ***: $p < 0.001$, **: $p < 0.01$.

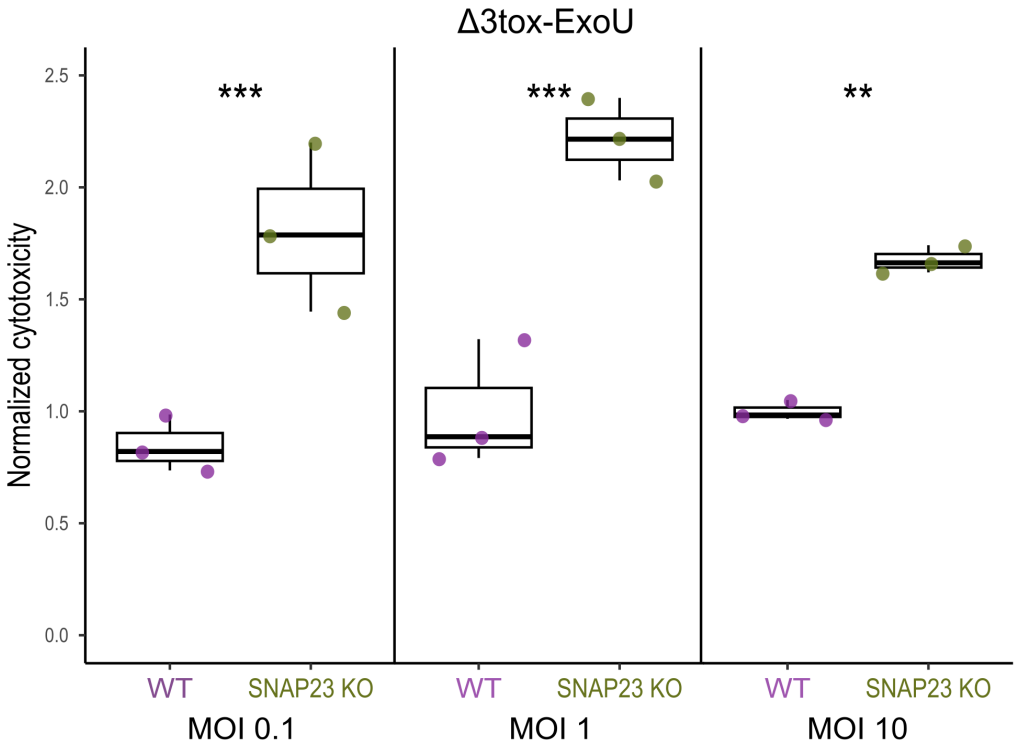

Fig S5. The SNAP23 KO sensitivity phenotype is independent of the MOI. WT or SNAP23 KO HEK cells infected with Δ3tox-ExoU *Pa* strains at increasing MOIs (0.1, 1 and 10). Cell death was monitored by live-cell imaging and data was normalized. The AUC of each condition is presented. WT cells are shown in purple, KO cells in green and individual values are represented as dots. ***: $p < 0.001$, **: $p < 0.01$.

Supplementary material and methods

Luminescence assay for intracellular cGMP production

Cells from the stable cell line expressing the cGMP-GloSensor reporter (1) were plated at 15 000 cells per well for two days in a white 96-well plate (Corning, 3610). Cells were grown in RPMI 1640 medium (Gibco, 61870010) supplemented with 10% heat-inactivated FBS and 200 µg/mL Geneticin (Gibco, 10131027) at 37°C and 5% CO_2. Bacterial cultures were prepared one day before infection by inoculating LB with the PAO1Δ3tox or PAO1Δ3tox-ExoY-UltraID *Pa* strain and grown overnight at 37°C, 180 rpm. The next day, overnight cultures were diluted in fresh LB and grown at 37°C, 200 rpm until they reached an OD_{600} of 1. Then, for each condition, 10.10^6 bacteria were resuspended in 1 mL of RPMI without red phenol (Gibco, 32404014) containing GlutaMAX (Gibco, 35050038). Cells were infected at delayed time points to allow simultaneous luminescence measurement to infect the wells, the cell culture

medium was removed from the corresponding well and replaced with 100 µL of bacterial suspension (MOI 20). After infection, 40 µL of a mixture containing 500 µL of GloSensor cAMP reagent (Promega, E1291) and 3.5 mL of RPMI without red phenol containing GlutaMAX was added in each well and luminescence was acquired luminescence every 3 minutes for 2.5 hours at 24–30°C using a TECAN Spark 10M plate reader. Data represent the maximum luminescence over time averaged from 8 technical replicates per strain.

Genetic manipulation for ExoS/T/Y-UltraID fusion proteins

Constructs were synthesized by Genewiz and cloned into pUC-GW-Amp plasmid. Each plasmid was digested by *HindIII* and *BamHI* and the fragment of interest cloned into digested miniCTX plasmid. The miniCTX plasmids were introduced into *P. aeruginosa* by triparental mating, using pRK600 as a helper plasmid. *P. aeruginosa* was selected on LB plates containing 10 µg/mL irgasan and 75 µg/mL tetracycline.

Supplementary tables

Table S1

***P. aeruginosa* strains**			
Strain	**Features**	**Reference/ origin**	**Name used**
PA14	ExoU^{+}	(2)	PA14
IHMA879472	T3SS^{-}, ExlA^{+}	(3)	IHMA
PP34	ExoU^{+}	(4)	PP34 WT
PP34Δ*exoU*	ExoU^{-}	(5)	PP34Δ*exoU*
CHAΔ*exoSexoT*	ExoS^{-} and ExoT^{-}	(6)	
CHAΔ*exoSexoT*//pUCP20 ExoU^{S142A} SpcU	ExoS^{-}, ExoT^{-} and ExoU^{+} catalytically inactive; Cb^{R}	(7)	CHA-ExoU^{A142}
PAO1Δ*exoTexoY*	ExoT^{-} and ExoY^{-}, ExoS^{+}	(8)	ΔTY-ExoS
PAO1Δ*exoSexoTexoY*	ExoS^{-}, ExoT^{-} and ExoY^{-}	(8)	PAO1Δ3tox
PAO1Δ*exoSexoTexoYpopBpopD*	ExoS^{-}, ExoT^{-}, ExoY^{-}, popB^{-} and popD^{-} (T3SS translocators)	Lab collection	
PP34ΔexoU::pminiCTX ExoU-UltraID	ExoU-UltraID^{+}, catalytically active, Tc^{R}	This work	ExoU-UltraID
PP34ΔexoU::pminiCTX ExoU*-UltraID	ExoU*-UltraID^{+}, catalytically inactive, Tc^{R}	This work	ExoU*-UltraID
PP34ΔexoU::pminiCTX ExoU-BioID2	ExoU-BioID2^{+}, catalytically active, Tc^{R}	This work	ExoU-BioID2
PP34ΔexoU::pminiCTX ExoU*-BioID2	ExoU*-BioID2^{+}, catalytically inactive, Tc^{R}	This work	ExoU*-BioID2

PP34ΔexoU::pminiCTX ExoU-iFAST	ExoU-iFAST^{+}, catalytically active, Tc^{R}	This work	ExoU-iFAST
PP34ΔexoU::pminiCTX ExoU*-iFAST	ExoU*-iFAST^{+}, catalytically inactive, Tc^{R}	This work	ExoU*-iFAST
PAO1Δ3tox//pUCP20 ExoU-UltraID SpcU	ExoU-UltraID^{+}, catalytically active; Cb^{R}	This work	
PAO1Δ3tox//pUCP20 ExoU*-UltraID SpcU	ExoU*-UltraID^{+}, catalytically inactive; Cb^{R}	This work	Parental *Pa*
PAO1Δ3toxΔpopBD//pUCP20 ExoU-UltraID SpcU	ExoU-UltraID^{+}, catalytically active; Cb^{R}	This work	
PAO1Δ3toxΔpopBD//pUCP20 ExoU*-UltraID SpcU	ExoU*-UltraID^{+}, catalytically inactive; Cb^{R}	This work	*ΔpopBD Pa*
PAO1Δ3tox::pminiCTX ExoS-UltraID	ExoS^{-}, ExoT^{-} , ExoY^{-} and ExoS-UltraID^{+}, Tc^{R}	This work	ExoS-UltraID
PAO1Δ3tox::pminiCTX ExoT-UltraID	ExoS^{-}, ExoT^{-} , ExoY^{-} and ExoT-UltraID^{+}, Tc^{R}	This work	ExoT-UltraID
PAO1Δ3tox::pminiCTX ExoY-UltraID	ExoS^{-}, ExoT^{-} , ExoY^{-} and ExoY-UltraID^{+}, Tc^{R}	This work	ExoY-UltraID
PAO1Δ3tox::pminiCTX ExoU SpcU	ExoS^{-}, ExoT^{-}, ExoY^{-} and ExoU^{+}, catalytically active, Tc^{R}	This work	Δ3tox-ExoU
***E. coli* strains**			
One Shot™ TOP10 Chemically Competent *E. coli*	Cloning strain	Invitrogen C404003	
Plasmids			
pet-15b-ultraID	Amp^{R}	Addgene 172879	
pcDNA3.1 myc-BioID2-MCS	Amp^{R}	Addgene 74223	
pAG247 His-iFAST	Kan^{R}	Addgene 130808	
pminiCTX	Tc^{R}		
pRK600	Helper plasmid used for conjugation, Cm^{R}	(9)	
pUCP20 *exoU^{S142A}-bla*	Amp^{R}	(5)	
pUCP20 *exoU-bla*	Amp^{R}	Lab collection	
pUCP20 ExoU*-BioID SpcU	Amp^{R}	Lab collection	
pUCP20 ExoU-UltraID	Amp^{R}	This work	
pUCP20 ExoU*-UltraID	Amp^{R}	This work	
pUCP20 ExoU-BioID2	Amp^{R}	This work	
pUCP20 ExoU*-BioID2	Amp^{R}	This work	
pUCP20 ExoU-iFAST	Amp^{R}	This work	
pUCP20 ExoU*-iFAST	Amp^{R}	This work	
pminiCTX ExoU-UltraID	Tc^{R}	This work	
pminiCTX ExoU*-UltraID	Tc^{R}	This work	
pminiCTX ExoU-BioID2	Tc^{R}	This work	
pminiCTX ExoU*-BioID2	Tc^{R}	This work	
pminiCTX ExoU-iFAST	Tc^{R}	This work	

pminiCTX ExoU*-iFAST	Tc^R	This work	
pUCP20 ExoU-UltraID SpcU	Amp^R	This work	
pUCP20 ExoU*-UltraID SpcU	Amp^R	This work	
pminiCTX ExoU SpcU	Tc^R	Lab collection	
pUC-GW-ExoS-UltraID	Amp^R	Genewiz	
pUC-GW-ExoT-UltraID	Amp^R	Genewiz	
pUC-GW-ExoY-UltraID	Amp^R	Genewiz	
pminiCTX ExoS-UltraID	Tc^R	This work	
pminiCTX ExoT-UltraID	Tc^R	This work	
pminiCTX ExoY-UltraID	Tc^R	This work	

Amp : ampicillin, Tc : tetracycline, Kan : kanamycin, Cb : carbenicillin

Table S2

	Primers sequence for SLIC	
Plasmid	**Forward (5'-3')**	**Reverse (5'-3')**
pUCP20 ExoU-UltraID + pUCP20 ExoU*-UltraID	aataaggagttcacatctagaggatcaggagacttcaagaacctgatctggc	acgacggccagtgccaagcttcacttctccttgaacttcttcag
pUCP20 ExoU-BioID2 + pUCP20 ExoU*-BioID2	aataaggagttcacatctagaggatcaggattcaagaacctgatctggctgaag	acgacggccagtgccaagctttagcttcttctcaggctgaactcg
pUCP20 ExoU-iFAST + pUCP20 ExoU*-iFAST	aataaggagttcacatctagaggatcaggagagcatgttgcctttggcagtg	acgacggccagtgccaagctttacacccgttcacaaagaccc
pUCP20 ExoU-UltraID SpcU	gcttggcggaataaggagttcactagtgaacaaaaactcatctcagaag	taaaacgacggccagtgccaagcttatcaacgaacactaacgcgctggt
pUCP20 ExoU*-UltraID SpcU	cttggcggaataaggagttcactagtgaacaaaactcatctcagaagag	gtatcaatcatttaactagtcttctccttgaacttcttcagg

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Chapter 6 - Supplementary results of the thesis

In this chapter will be presented a set of experiments that were performed for various aims and gave results that did not allow us to go further or provided preliminary results as basis for future experiments. In both cases, these experiments provided answers for important questions and choices throughout my thesis. These results will be discussed in the following **General discussion** chapter.

6.1 Engineered ExoU-APEX2 is not secreted through the T3SS

We engineered the ExoU-APEX2 fusion protein similarly to the BioID2, UltraID and iFAST constructs and expressed it in a PP34Δ*exoU* strain (**Figure 30 A**). The aim was to carry out proximity labeling using APEX2 as it is able to provide a biotinylation signal upon 1 min of activation with H_2O_2, allowing us to perform kinetics experiments. We tested the concerned strain for the *in vitro* secretion of ExoU-APEX2 by the T3SS using Ca^{2+} chelation by EGTA. In contrast to the other validated constructs, the ExoU-APEX2 fusion was exclusively detected in the bacterial pellet but not in the respective supernatant (**Figure 30 B**). Therefore, further work with APEX2 was stopped at this stage.

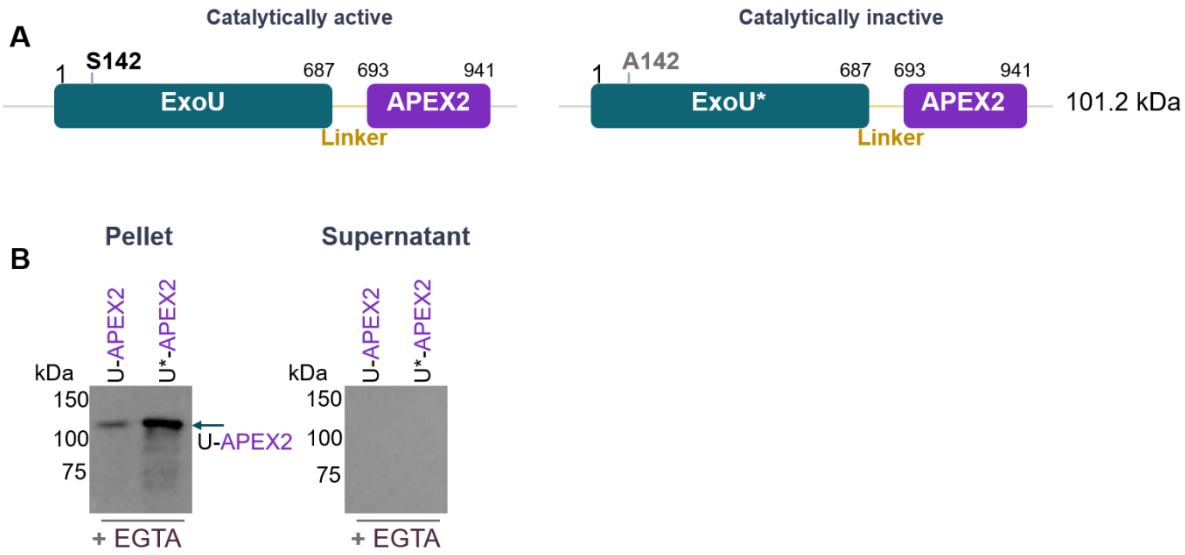

Figure 30. Engineered ExoU-APEX2 fusion proteins are not secreted.
A. Schematic representation of the ExoU-APEX2 fusion proteins constructs inserted into the genome of a Δ*exoU* strain. Active ExoU (S142) and inactive ExoU* (A142) fusions were generated. A linker (SRGSG) separates ExoU and APEX2. **B**. Western blot showing the expression of the fusion proteins and their secretion in the supernatant upon an *in vitro* induction of the T3SS (+EGTA). Western blot was revealed with α-ExoU antibody.

6.2 Possibility of biotinylation kinetics during infection

The idea behind performing proximity labeling in kinetics is to be able to distinguish specific ExoU proxisomes at different stages of its intracellular journey. As it was not possible to execute kinetics experiments using APEX2, we performed PLA using a short infection time

with strains injecting the ExoU*-UltraID and ExoU-UltraID in a PAO1Δ3tox background. Cells were simultaneously infected with bacteria and treated with biotin (500 µM) and then incubated for 1 hour. The cells were collected and processed as previously described. When revealing the WB with streptavidin-HRP, to detect biotinylated proteins, we observed a similar pattern to that of the 5.5 hours biotinylation reported in the draft of the publication. The biotinylated proteins were detected for both bacterial strains, wild-type and mutant ExoU, including a band at the molecular weight of the auto-biotinylated ExoU (**Figure 31**). This suggests that it is possible to biotinylate for 1 hour for both strains and to perform kinetics analysis by adding biotin at different times post-infection. This applies only for the mutant strain as the wild-type strain causes cell death starting 2.5 hours post-infection. In this case, the kinetics could be extended until 2.5 hours at the latest. This would allow us to determine if there are any differences in the ExoU proxisome between the wild-type and mutant ExoU. Unfortunately, we did not have time to perform these kinetics experiments and preferred to focus on control conditions for the PLA carried out at 5.5 hours post-infection.

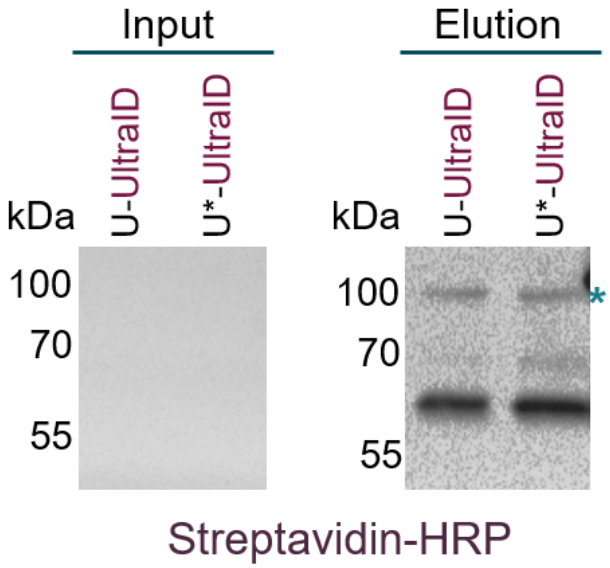

Figure 31. The one-hour PLA supports conducting kinetics experiments.
Western blot was revealed with streptavidin-HRP showing the enrichment of biotinylated proteins after a 1 hour PLA using A549 WT cells. Cells were infected, for 1 hour, either with the ExoU-UltraID or ExoU*-UltraID *Pa* strain and biotin was simultaneously added at 500 µM. Auto-biotinylated ExoU*-UltraID ~100 kDa is indicated by an *.

6.3 Attempt to track injected ExoU by iFAST

The ExoU*-iFAST fusion, used as a negative control for the PLA, was initially constructed to track injected ExoU within host cells. iFAST is a protein that docks its exogenous ligand (fluorogen) in an emissive conformation allowing the tracking of the protein by fluorescence microscopy (Tebo et al., 2018). In the context of bacterial infections, it was used to investigate the intracellular replication of *Listeria monocytogenes* in eukaryotic cells (Peron-Cane et al., 2020) .To test the possibility of the use of iFAST, we infected A549 WT cells with the ExoU*-

BioID2 strain as a negative control and with the ExoU*-iFAST expressing strain. The samples were treated with the exogenous ligand prior to acquisition. In the BioID2 condition, we observe a background signal that could be due to the presence of the free ligand. Whereas in the iFAST condition, a specific signal corresponding to the bacteria is detected in addition to a weak signal in the A549 cells, that is somehow more prominent and differently distributed compared to the BioID2 sample (**Figure 32**). This implies that the protocol needs to be optimized (ligand concentration and duration of infection) to yield an improved and more specific signal.

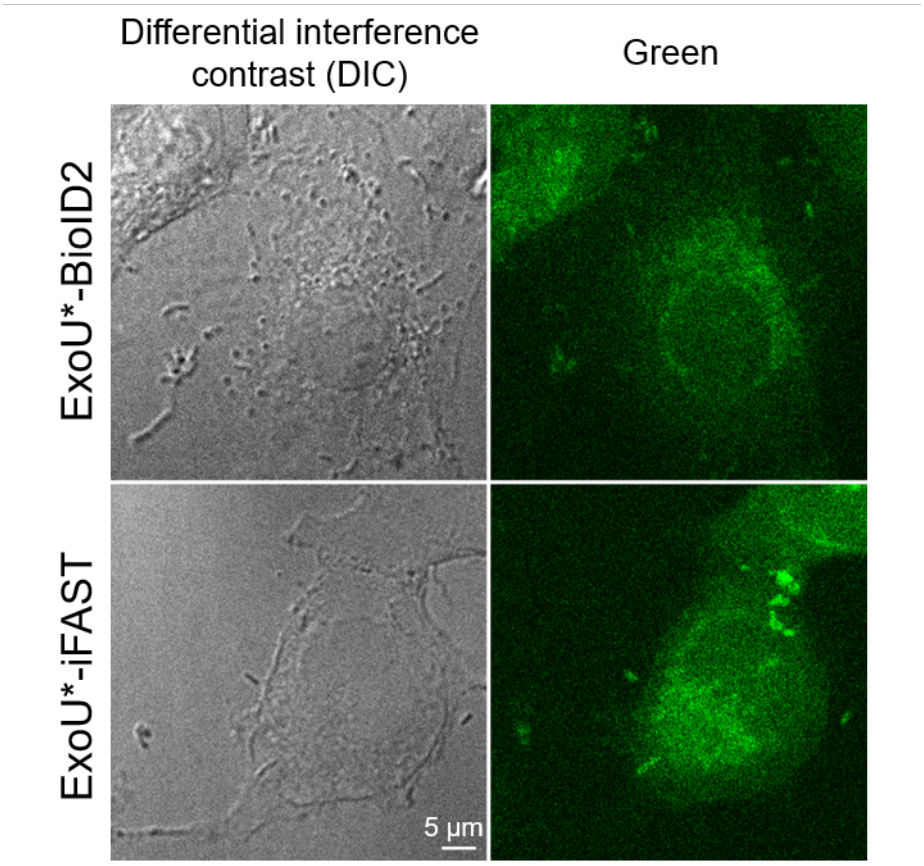

Figure 32. First fluorescent labeling of ExoU*-iFAST.
A549 WT cells were infected for 20 minutes with a *Pa* strain injecting ExoU*-iFAST or ExoU*-BioID2. The green fluorogen was added for both conditions just before image acquisition at a final concentration of 5 µM. Fields were imaged using differential interference contrast (DIC) and the green channel was used to assess the iFAST signal.

6.4 Induction of exosomes secretion during HEK WT cells infection

We wondered whether the infection by an ExoU-positive strain would induce CD63-decorated EVs secretion in HEK WT cells. We took advantage of the engineered HEK WT cells that express HA-tagged CD63 fused to the NanoLuciferase. This cell line was developed to provide an easy and sensitive way to quantify secretion in the supernatant of cultured cells while

maintaining basal EVs secretion and biogenesis. The NanoLuc system has been used as a luminescent reporter to detect the presence of the POI fused to the luciferase.

A first assay of NanoLuc measurements in the supernatant of cells infected for 7 hours (MOI 10) detected no difference between the two bacterial strains used, Δ3tox and Δ3tox-ExoU^{S142A}. In addition, the values were similar to the ones measured in the non-infected cells supernatant (**Figure 33 A**). Matching results were obtained with a 16-hour infection at MOI 0.01 (**Figure 33 B**). This shows that in the tested conditions, ExoU does not affect the secretion of exosomes by HEK WT cells. Lastly, the signal detected in the supernatant of non-infected cultured cells at 7 hours of incubation is around half of what is observed at 23 hours (**Figure 33 C**). This suggests that the secretion of CD63 EVs slows down over time, as the observed luminescence increase is not proportional (7 *vs* 23 hours).

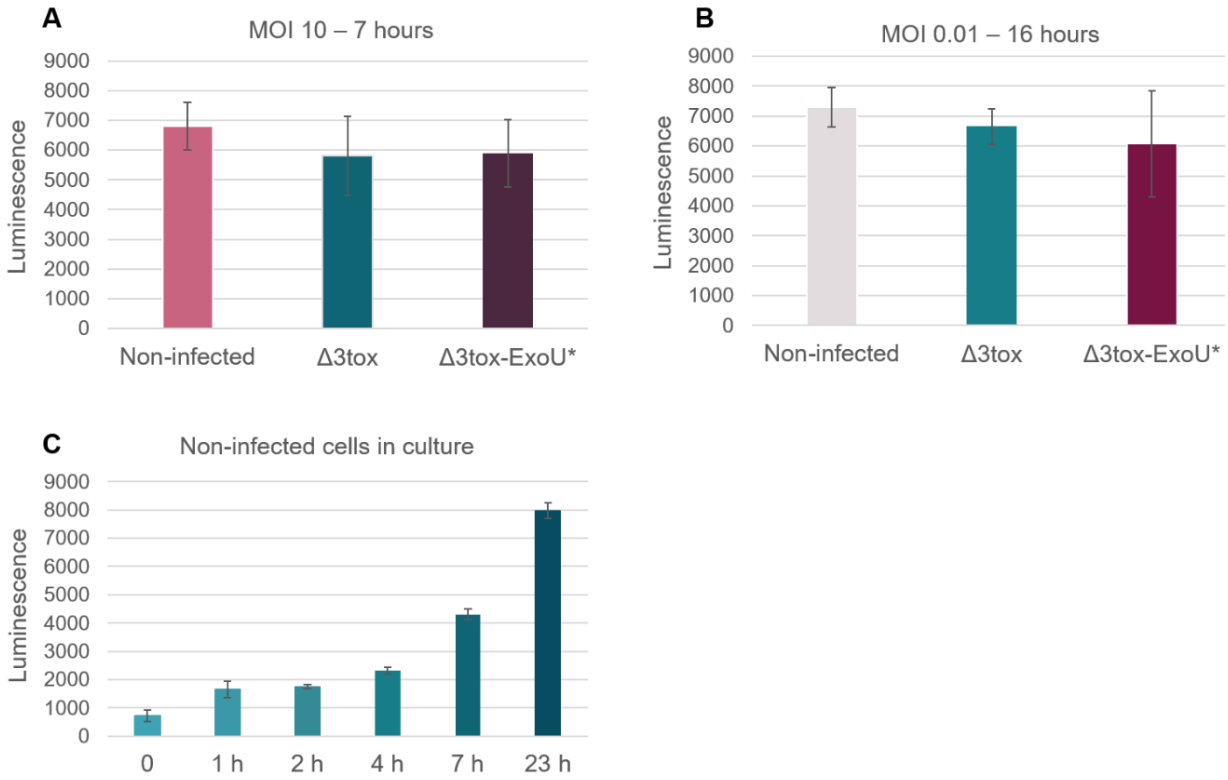

Figure 33. CD63-positive EVs secretion in HEK WT cells.
A. HEK WT cells were infected or not with a Δ3tox and Δ3tox-ExoU^{S142A} *Pa* strains at MOI 10. The Nanoluc measurements in the supernatants of infected cells were performed at 7 hours post-infection and compared to the non-infected cell supernatant. **B**. HEK WT cells were infected or not with a Δ3tox and Δ3tox-ExoU^{S142A} *Pa* strains at MOI 0.01. The Nanoluc measurements in the supernatants of infected cells were performed at 16 hours post-infection and compared to the non-infected cells' supernatant. **C**. Nanoluc measurements, at different time points, in the supernatants of non-infected cells growing over 23 hours.

6.5 Levels of the proteins of interest in the used cell lines

In a simple test, we determined the levels of the hits obtained from PLAs (SLC3A2, SNAP23 and RAB27B) in the different cell lines used during the project. Based on protein quantification, similar§§ quantities of the lysates of each cell line: A549 WT, DNAJC5$^{-/-}$ (KO) and A549 cells complemented with FLAG tagged DNAJC5 (C) – fibroblasts WT and SLC3A2 KO cells - HEK WT and SNAP23 KO cells were analyzed by immunoblotting using the corresponding antibodies. We observed that RAB27B and SLC3A2 were only detected in the A549 cell lines (**Figure 34 A and C**). The A549 WT cells contain more SLC3A2 than their derivatives DNAJC5$^{-/-}$ and complemented. It is to note that the SLC3A2 in the fibroblasts were not detected as the antibody used was against the human form of SLC3A2 and the fibroblasts are from mice (**Figure 34 A**). The SNAP23 protein was identified in all cell lines except the HEK SNAP23 KO cells (**Figure 34 B**). Slightly lower SNAP23 and RAB27B quantities are detected for the A549 WT and DNAJC5$^{-/-}$ cells compared to the complemented (**Figure 34 B and C**). This suggests that modifying the DNAJC5 expression (KO or overexpression upon complementation) could affect the other proteins' expression within this cell line. Mass spectrometry is an option for a more sensitive and accurate quantification of the different proteins especially the ones not detected by western blot.

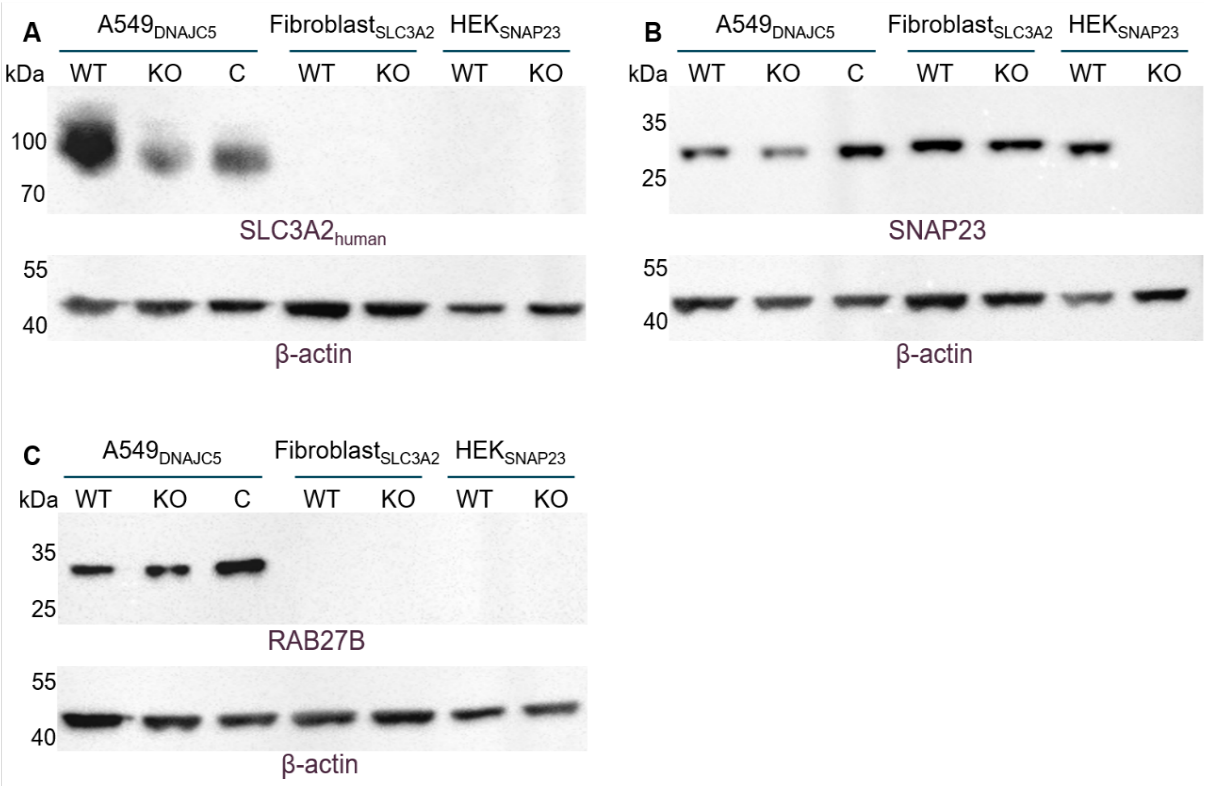

Figure 34. Comparison of the protein levels of SLC3A2, SNAP23 and RAB27B.
Western blot analysis was performed on lysates from A549 (WT and DNAJC5 KO), murine dermal fibroblasts (WT and SLC3A2 KO) and HEK (WT and SNAP23 KO) cells. Western blot was probed with α-human SLC3A2 (**A**), α-SNAP23 (**B**) and α-RAB27B (**C**) antibodies. Actin was used as a loading control.

6.6 Supplementary material and methods

6.6.1 Fluorescence microscopy – iFAST

A549 WT cells (50 000 cells/slide) were seeded 2 days prior to infection. The T3SS of the PP34ΔExoU::ExoU*-BioID2 and PP34ΔExoU::ExoU*-iFAST strains was pre-induced *in vitro* with EGTA. Cells were then infected with either of the strains at MOI 100 (ExoU*-BioID2) or MOI 10 (ExoU*-iFAST) for 15 minutes. After infection, cells were fixed for 30 minutes with 4% paraformaldehyde (PFA). Piror to acquisition, slides were incubated with the ^{TF}Lime membrane-permeant fluorogenic ligand (The Twinkle Factory – 480541-250) at a final concentration of 5 µM. Cells were imaged using differential interference contrast (DIC) and the fluorescence green channel. Images were acquired on a spinning disk confocal microscope (Olympus IX81, GATACA systems, Andor iXon EMCCD camera) equipped with a 100× oil immersion objective. Image analysis was performed using Fiji software.

6.6.2 Luminescence assay for CD63-decorated exosomes secretion

HEK WT cells were seeded (20 000 cells/well) one day prior to the experiment in a 96-well plate. Before infection, the medium was replaced with DMEM 10 % FBS without red phenol. Cells were infected (or not) with the PAO1Δ3tox and PAO1Δ3tox-ExoU^{S142A} at MOI 10 and 0.01. Supernatants were collected at 7 or 16 hours post-infection from samples infected (or not) at MOI 10 and 0.01, respectively. For the non-infected samples, supernatants were collected just after the medium was changed (t0) and at 1, 2, 4, 7 and 23 hours of growth. Supernatants were centrifuged at 3000 g for 5 minutes before analysis. The Nano-Glo Luciferase assay substrate was diluted 1:50 in the buffer according to the manufacturer's instructions (Promega N1110). The substrate was then mixed with the supernatant at a 1:1 ratio and luminescence was measured using the Clariostar Plus (BMG LABTECH) plate reader. This experiment was performed in triplicates.

General discussion

ExoU is one of the most potent *P. aeruginosa* toxins and it has been intensively studied in the context of infections and host-pathogen interactions. Different aspects of ExoU were investigated including its interactions within the bacteria such as with its chaperone SpcU, 3D structure, enzymatic activity, importance during infections, effect on the immune response, interaction with the eukaryotic host cofactors and its intracellular trafficking (as described in chapter 2).

During my thesis, I optimized and applied proximity labeling, using a biotin ligase, on a T3SS injected toxin of an extracellular pathogen. Most bacterial studies either express the fusion protein within the eukaryotic cell or express it in intracellular pathogens replicating within vacuoles (Mousnier et al., 2014; Mojica et al., 2015). With the aim to better understand the trafficking of ExoU, the PL allowed us to identify host proteins maneuvering the trafficking as a part of the cellular response towards the ExoU injected in the host cytoplasm. The knock-out cell lines of two host factors pointed out by PL, SLC3A2 and SNAP23, rendered the cells more sensitive to the necrotic activity of ExoU which led us to propose a protective role for these proteins against ExoU during infection. This is the first report in which host factors are proposed to protect against a *P. aeruginosa*–injected effector, with their protective role also being revealed through PL.

Throughout the project, we encountered several technical difficulties that are informative for future studies, some of which could be potentially overcome through further optimization. The ExoU-APEX2 fusion was not secreted by the T3SS which, once again, points towards the T3SS selectivity towards the secreted substrates (Notti & Stebbins, 2016). In this case, it could be that APEX2 is unable to properly unfold and therefore is not processed by the T3SS machinery, similarly to what is observed for the GFP fusion (Akeda & Galán, 2005). Also, it is possible that the APEX2 fusion is “stuck” within the secretion machinery or the needle. It would be hard to discriminate between the two possibilities as in both cases, the secretion of other T3SS effectors is reduced (Riordan et al., 2008; Dohlich et al., 2014). Although not investigated in this work, it would be interesting to directly transfect the eukaryotic cells with a plasmid encoding the APEX2 fusion because of the short labeling time advantage it offers (Hung et al., 2016). This approach is often applied for secretion systems effectors (Mousnier et al., 2014; D’Costa et al., 2019). However, it presents disadvantages including the artificial high levels of fusion proteins that could lead to false positives in addition to that the effector is not taking its natural route of injection into host cells therefore interesting hits could be missed.

As much as it is tempting to perform kinetics using the ExoU*-UltraID *P. aeruginosa* strain by adding biotin at different times of infection, it could be quite tricky. It is important to synchronize the infections across different conditions. This can be achieved through an *in vitro* pre-induction

of the T3SS, a centrifugation step just after infection (Jiang et al., 2021) and cell cycle synchronization by fetal bovine serum starvation (FBS) (He et al., 2010). These steps help ensure that cells are at a comparable stage of infection; otherwise, the populations would be heterogeneous which could introduce bias into the results.

During my work, I have shown that ExoU could be detected in EVs. The impact of ExoU injection on the secretion of CD63-positive EVs can be further investigated. First, for the CD63-NanoLuc secretion assay, it is essential to optimize the infection conditions (MOI and length of infection) in addition to including a normalization to the intracellular CD63 or to the number of cells per well, as infected cells do not divide at the same rate of non-infected cells (Leitão et al., 2014; Sol et al., 2019). Three scenarios are conceivable; an increase of secretion is induced by infection independently of any exotoxin, the increase is specific to ExoU or no increase is observed as ExoU is handled by host proteins expressed at basal levels. It is also possible to overexpress or downregulate CD63 to increase or decrease secretion levels and investigate whether we observe a difference in the sensitivity to the activity of ExoU. Concerning the isolated EVs, they can be subjected to further characterization. A first thing would be to determine if ExoU is present within the same EVs population as CD63, which could be done through a specific kit that exclusively purify CD63 decorated vesicles. Tryptic digestion of the isolated veiscles would allow us to determine if ExoU is on the outside or the inside of these vesicles, as trypsin can only digest extravesicular cargo (Choi et al., 2020). We found no evidence in the literature that ExoU has ever been detected in the outer membrane vesicles (OMVs) of *P. aeuginosa* which is in accordance with our hypothesis that ExoU is in host cell-derived vesicles.

When it comes to the live tracking of ExoU during infection, it remains a major experimental challenge. The iFAST was used to study the vacuolar life cycle of *Listeria monocytogenes* and its replication within vacuoles. It was observed that when iFAST efficiently accumulated, it could be distinguished from the non-specific signal but once the vacuole ruptured, the signal drastically dropped (Peron-Cane et al., 2020). In our case, we could be facing a similar issue as ExoU is diluted within the host cytoplasm (punctuate pattern with α-ExoU) and mostly accumulates at the PM (Deruelle et al., 2021) which is an obstacle for particle tracking. Further tests should be performed to determine whether iFAST can be ruled out or considered as a possible tool to track ExoU.

An alternative worth exploring is the use of self-labeling enzymes (SLEs) such as the HaloTag, SNAP-tag and CLIP-tag. The substrates of SLEs are coupled to fluorochromes and in the presence of the substrate, SLEs catalyze a reaction where they become bound to the substrate and therefore fluoresce. They have been tested and validated on T3SS translocated effectors

of the intracellular pathogens *Salmonella enterica* serovar Typhimurium and *Yersinia enterocolitica* (Göser et al., 2019) thus they could be viewed as a viable option for live ExoU tracking.

The mystery of the identity of the phosphoinositides (PIPs) composing the vesicles carrying ExoU within host cells remain unsolved. Lipid sensors have been specifically designed to identify and mark different phosphonositides within eukaryotic cells. The biosensors were fused to the SNAP tag which could be coupled to different fluorochromes and validated in eukaryotic cells (Maib et al., 2024). It is possible to combine these sensors with the anti-ExoU labeling and subsequently reveal the lipid composition of ExoU's environment. This is important because it remains unclear how ExoU anchors to the vesicles' membrane (*via* interaction with lipids or a specific receptor) and which ExoU domain mediates this interaction. In addition, it would be interesting to identify the different compartments ExoU localizes to at different times of infection. This can be done through fluorescence microscopy labeling of ExoU and known well-defined compartments by using their respective markers to help better understand its trafficking.

The structure of ExoU bound to its eukaryotic host factors, PI(4,5)P2 and ubiquitin, remains to be elucidated. This would permit the visualization of the real conformational changes occurring within the domains of ExoU upon its interaction with these two essential cofactors. This might also point to the role of the ubiquitination at the Lys178 residue of ExoU. While the mutation of this residue does not affect the activity of ExoU, it disrupts its localization with the early endosome marker (EEA1) (Gendrin et al., 2012). The precise relevance of this modification is still unclear, as it may be involved in the trafficking, conformational modification or the processing of ExoU within the host cell.

Lastly, other identified hits in the MS including RAB27B could be similarly investigated, as we might not be looking at a sole ExoU detoxification pathway. Additionally, some hits might actually favor ExoU activity as observed for DNAJC5 (Deruelle et al., 2021). Regarding the RAB27B, we have just received the KO cells that we have been waiting for over the past ten months.

Overall, the PL approach revealed unprecedent insights about the whereabouts of ExoU during eukaryotic cell infection which illustrates complex host-pathogen interplay. Despite ExoU being studied for more than 20 years now, significant gaps remain in our understanding of ExoU's structure and its dynamics within the host. Hence, this highlights ExoU's potential as a subject for future studies.

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Summary

Mapping the Host Proxisome of ExoU, a Type III Secretion System effector of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*Pa*) is a multidrug-resistant and a major human Gram-negative opportunistic pathogen able to cause a variety of infections. It assembles on its surface an arsenal of virulence factors including the potent Type 3 Secretion System (T3SS) which injects four toxins into eukaryotic cells. Among them, the most aggressive toxin, the phospholipase ExoU targets the host plasma membrane (PM) leading to a rapid necrotic cell death. Recently, our team identified the host chaperone DNAJC5, critical for the Misfolding-Associated Protein Secretion (MAPS) pathway, as essential for targeting ExoU to the PM. However, other host factors governing the intracellular journey of ExoU remain unknown.

In this study, we employed proximity labeling (PL) and integrated cellular approaches, to better understand the crosstalk between ExoU and the host's trafficking machinery. To do so, we used the biotin ligase, UltraID as PL tool. We expressed ExoU fused to UltraID in *Pa* and performed PL assays during infection. In the presence of exogenous biotin, UltraID mediates the biotinylation of proteins in close proximity to ExoU. The resulting proteins are then captured by streptavidin and undergo tryptic digestion followed by LC-MS/MS. Analysis revealed nine significantly enriched human proteins when ExoU-UltraID is injected, among them SNAP23 and SLC3A2, which we prioritized due to their known roles in trafficking and/or DNAJC5-dependent pathways. Cells that lack either of these two proteins showed increased sensitivity to an infection with ExoU^{+} strain. The mutants and their parental cell lines were similarly susceptible to other T3SS (ExoS) and PM-damaging (ExlA) toxins indicating an ExoU specific phenotype. In addition, immunofluorescence revealed a partial colocalization of ExoU with SLC3A2 and CD63, marker of extracellular vesicles (EVs). Given that SNAP23 mediates the exocytosis of CD63-positive vesicles, we hypothesized that ExoU is detoxified *via* this pathway. Consistent with this, ExoU was detected in isolated EVs from the supernatant of infected cells. For the first time, we applied PL to a T3SS injected toxin of an extracellular pathogen. We identified host factors taking part in the host cell response to ExoU, underlining a complex host-ExoU interplay.

Keywords : *Pseudomonas aeruginosa*, proximity labeling, ExoU, trafficking, SLC3A2, SNAP23

Résumé

Cartographie du Proxisome d'ExoU, un Effecteur du Système de Sécrétion de Type III de *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*Pa*) est un pathogène opportuniste Gram-négatif multirésistant et majeur chez l'homme, capable de provoquer diverses infections. *Pa* présente à sa surface un arsenal de facteurs de virulence, dont le puissant Système de Sécrétion de Type 3 (SST3) qui injecte quatre toxines dans les cellules eucaryotes. Parmi celles-ci, la toxine la plus agressive, la phospholipase ExoU, cible la membrane plasmique (MP) de l'hôte, entraînant une nécrose cellulaire. Notre équipe a montré que la protéine de l'hôte DNAJC5, de la voie de sécrétion MAPS, est essentielle à l'adressage d'ExoU à la MP. Cependant, d'autres facteurs de l'hôte guidant le parcours intracellulaire d'ExoU restent inconnus.

Dans ce projet, nous avons utilisé le marquage de proximité (PL) et des approches cellulaires intégrées afin de mieux comprendre la relation entre ExoU et la machinerie de trafic de l'hôte. Pour cela, nous avons utilisé la biotine ligase UltraID comme outil de PL. Nous avons exprimé ExoU fusionnée à UltraID dans *Pa* et avons réalisé des tests de PL pendant l'infection. En présence de biotine exogène, UltraID permet la biotinylation des protéines à proximité d'ExoU. Les protéines ainsi obtenues sont ensuite capturées par la streptavidine et subissent une digestion tryptique suivie d'une analyse par LC-MS/MS. L'analyse a révélé 9 protéines humaines significativement enrichies quand ExoU-UltraID est injectée, parmi lesquelles SNAP23 et SLC3A2, priorisées en raison de leurs rôles connus dans le trafic et/ou les voies dépendantes de DNAJC5. Les cellules dépourvues de l'une ou l'autre de ces deux protéines ont montré une sensibilité accrue à une infection par la souche ExoU^{+}. Les mutants et leurs lignées cellulaires parentales présentaient une sensibilité comparable envers d'autres toxines du SST3 (ExoS) et des toxines endommageant la MP (ExlA), ce qui indique un phénotype spécifique à ExoU. De plus, l'immunofluorescence a révélé une colocalisation partielle d'ExoU avec SLC3A2 et CD63, un marqueur des vésicules extracellulaires (EVs). Étant donné que SNAP23 intervient dans l'exocytose des vésicules CD63-positives, nous avons émis l'hypothèse qu'ExoU est détoxifiée *via* cette voie. Conformément à cette hypothèse, ExoU a été détectée dans des EVs isolées à partir du surnageant de cellules infectées. Pour la première fois, nous avons appliqué le PL à une toxine injectée par le T3SS d'un pathogène extracellulaire. Nous avons identifié des facteurs de l'hôte participant à la réponse de la cellule hôte à ExoU, soulignant une dynamique complexe entre l'hôte et ExoU.

Mots clés : *Pseudomonas aeruginosa*, marquage de proximité, ExoU, trafic, SLC3A2, SNAP23