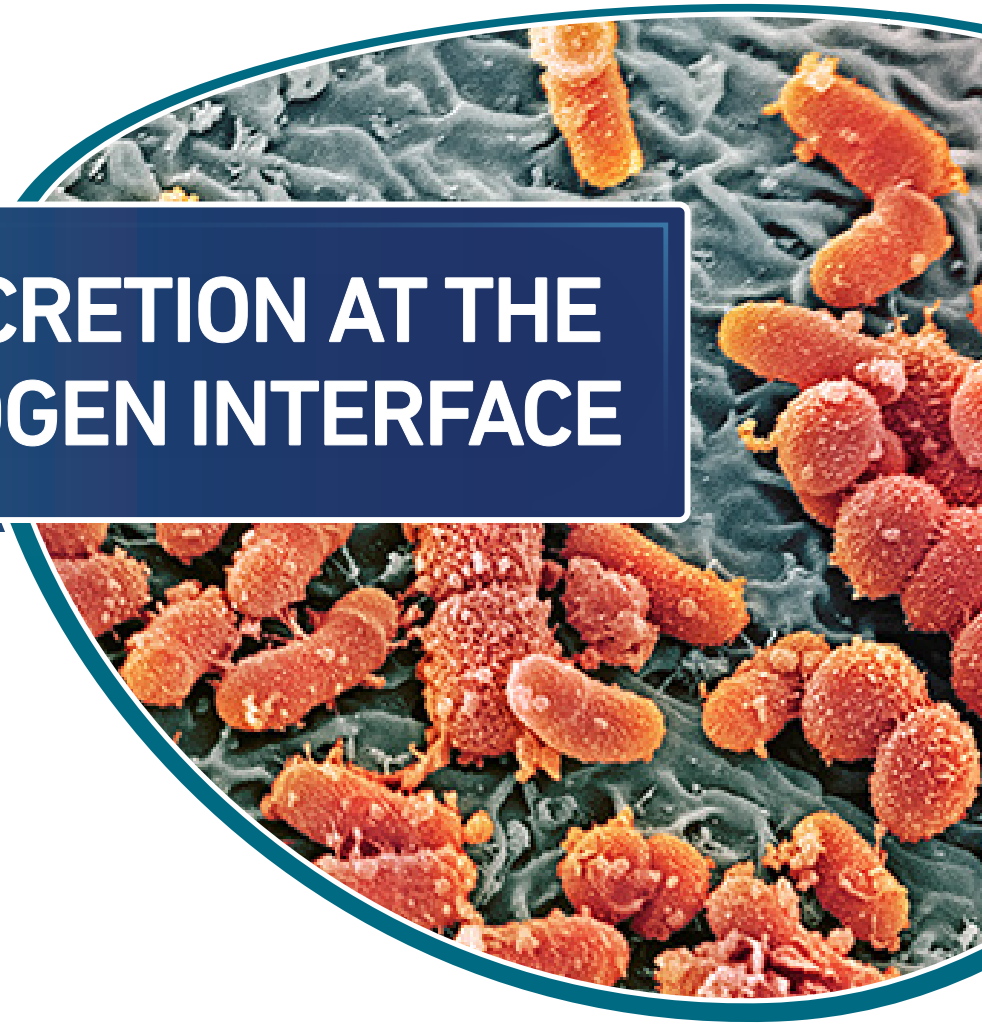
MICROBIOLOGY SOCIETY

FOCUSED MEETING
2022

PROTEIN SECRETION AT THE HOST-PATHOGEN INTERFACE



3–4 November 2022, Riddel Hall, Belfast, UK

Abstract book

Poster #1

Control of type VI secretion system positioning in Enteroaggregative *E. coli*

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Abstract

The type VI secretion system (T6SS) is an efficient weapon used by bacteria to outcompete other bacteria and to colonise a niche. This nanomachine can be compared to a molecular speargun and allows bacteria to secrete effectors into the extracellular environment or directly into target cells in a contact-dependent manner. The core components as well as the mechanism of action of this machinery are now well characterised. However, proteins with accessories function have been recently discovered. These proteins improve T6SS efficiency but are not essential for T6SS function. We recently identified a new accessory gene that we named tagI (for Type VI associated gene with Inhibitory function), whose deletion increases the number of T6SSs per cell. Using protein-protein interaction assays, we showed that TagI acts as post-translational inhibitor of T6SS tail polymerisation by interacting with a component of the tube/sheath complex. Fluorescence microscopy analyses of cells producing TagI-GFP demonstrated that TagI colocalises with the chromosome. This specific localisation is mediated by direct interaction between TagI and the DNA as shown by electrophoretic mobility shift and ChIP-Seq assays. Taken together, our results suggest that TagI inhibits T6SS assembly at the center of the cell to avoid obstruction by the chromosome during tail polymerisation.

Poster #2

The molecular basis of FimT-mediated DNA uptake during bacterial natural transformation

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Abstract

The emergence of antibiotic resistant bacteria is a major threat to human health and the rapid spread of this phenomena can be attributed to the process of horizontal gene transfer. One mechanism of horizontal gene transfer is natural transformation, accomplished by naturally competent bacteria. Bacterial natural transformation comprises the uptake and translocation of exogenous DNA into the cell. The type IV pilus is crucial for the initial uptake step in most naturally competent species. These proteinaceous cell-surface structures are composed of thousands of pilus subunits (pilins) with the ability to elongate and retract in an ATP-dependent manner. The relative abundance of pilins within the pilus designates these subunits as major or minor.

We identified the minor pilin FimT as an important component of *Legionella pneumophila's* type IV pilus during natural transformation. To understand the molecular basis of FimT's role, we determined the structure by NMR spectroscopy, performed *in vitro* DNA binding assays and *in vivo* transformation assays. Our data shows that FimT binds DNA via an electropositive patch, with a well conserved motif located in a conformationally flexible C-terminal tail. Orthologues of FimT are widely distributed amongst Gammaproteobacteria and we confirmed that those from *Pseudomonas aeruginosa* and *Xanthomonas campestris* share the ability to bind to DNA *in vitro*. Our findings suggest that a wide range of competent bacterial species use FimT as a DNA-receptor during natural transformation.

Poster #3

Characterization of the Type IX secretion pathway

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Abstract

Among bacterial secretion systems, the type IX secretion system (T9SS) possesses unique features and allows bacteria from the Bacteroidetes phylum to secrete a wide variety of substrates, from enzymes for biopolymers degradation, to virulence factors in the pathogen Porphyromonas gingivalis, or adhesins required for gliding motility in our model Flavobacterium johnsoniae.

T9SS substrates are secreted in two steps. They are first translocated across the inner membrane by the Sec machinery. Then they are recruited by the T9SS thanks to a conserved C-terminal domain (CTD) to be secreted through the outer membrane. Recently, an in situ cryo-electron tomography approach revealed the architecture of P. gingivalis T9SS. However, only little is known regarding the secretion mechanism and the interactions driving substrates along the components of the T9SS during secretion.

To decipher the precise pathway followed by effectors and to understand the role of each interaction in space and time, I am using a proximity labeling method. As proteins fused to a CTD can be secreted by the T9SS, I fused the APEX2 peroxydase to different CTDs. During its secretion, APEX2 will biotinylate the proteins interacting with the CTD. The biotin-labeled proxisome can then be analyzed by mass spectrometry.

The idea is to use a genetic approach with diverse T9SS mutants to identify the interactions that are essential for the CTDs to travel through the T9SS and to understand how they condition the following steps in the pathway. I will present some of our first results, highlighting the importance of the GldLM motor.

Poster #4

Copper tolerance systems in Pseudomonas aeruginosa : role in defense against phagocytosis

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Abstract

The opportunistic pathogen Pseudomonas aeruginosa can cause acute or chronic infections by using a whole arsenal of systems allowing its virulence. Among these mechanisms, the secretion systems play a determining role.

The Tat system, allowing the export of substrate proteins from the cytoplasm to the periplasm, is essential for the virulence of Pseudomonas aeruginosa1. Previous studies have shown that this system confers to P. aeruginosa a better tolerance to copper. During infection, copper is used by macrophages as an anti-bacterial agent2. Copper is an essential element for bacteria but can become toxic at high concentration. Its toxicity is due to its ability to compete with iron in the iron-sulfur centers of proteins and to induce oxidative stress.

The aim of my thesis is to better understand the role of the Tat system and its substrates in copper tolerance in Pseudomonas aeruginosa. Among the 34 proteins identified by the team as substrates of the Tat system3, we identified three that could be good candidates to explain the sensitivity of the tat mutant to copper. Indeed, a targeted approach allowed us to highlight a sequence homology between two of the 34 Tat substrates and copper resistance proteins of the Multi-copper oxidase family. In parallel, the screening of a library of Tat substrates mutants, revealed a third candidate: an enzyme involved in the biosynthesis of a siderophore.

My thesis work consists in studying the expression conditions of the three candidates, their role in copper resistance and in survival to phagocytosis.

Poster #5

Molecular characterization of two novel Brucella effectors

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Abstract

Brucella is responsible for a major zoonotic disease and relies on a type IV secretion system for intracellular proliferation. Recently, we identified two Brucella effectors that modulate the spatial localization of nuclear proteins to promote virulence. In this study, we characterized the biophysical properties of these effectors and the molecular interactions mediating their function.

We combined cellular (infection, transfection, imaging, co-immunoprecipitation) with biochemical (affinity, pull-down experiments) as well as biophysical (SEC-MALS-RI, MicroScale Thermophoresis) methods to characterize these Brucella effectors named NyxA and NyxB.

Size exclusion chromatography coupled to multi-angle light scattering and refractometry (SEC-MALS-RI) of NyxA and NyxB showed that both effectors behave as dimers in solution. Moreover, the crystallographic structure of NyxB provided insight of the dimerization interface formed between both Nyx monomers (Louche & al. BioRxiv 2021). We have now identified the key residues in the interface between NyxA and NyxB homodimers that were point-mutated. In vitro experiments using SEC-MALS-RI and SAXS confirmed the monomeric state of these mutants. We also characterized the Nyx effector interactions using pull-down approaches and MST analysis, demonstrating the formation of a heterodimer between NyxA/NyxB. The role of these interactions in infection will be presented. Lastly, we evaluate the impact of the NyxA-NyxB complex on the binding affinity to its cellular target, SENP3, a nucleolar deSUMOylase, which is involved in ribosomal biogenesis.

Overall, molecular characterization of NyxA and NyxB effectors is essential for defining how they modulate SENP3 activity and advancing our understanding of how Brucella manipulates host cell responses during infection.

Poster #7

Investigation of microbial community dynamics i*n vivo* using zebrafish infection models

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Abstract

Humans, and many other species, are colonised by a large, complex community of microbes called the microbiome. Interactions among members of the microbiome, as well as with host immune factors, influence the microbial ecosystem's structure, composition, and diversity. However, the nature of interbacterial interactions during *in vivo* infection remains unclear. For example, the Type 6 Secretion System (T6SS), a widely conserved, contact-dependent protein secretion system that mediates antagonistic cell-cell interactions in many Gram-negative bacteria, has been implicated in commensal killing. However, the spatial and temporal dynamics of T6SS-mediated antagonism *in vivo* have not yet been explored. We developed a *Vibrio cholerae* zebrafish infection model and used time-lapse fluorescent microscopy to study bacterial cell-cell interactions occurring within three-dimensional, multi-species, bacterial communities. We used our zebrafish infection model to characterise the role of T6SS-mediated bacterial antagonism during colonisation and pathogen invasion of pre-established communities of commensal bacteria. The influence of temperature, cell density and time interval between injections were explored to understand how these parameters effect T6SS-mediated interactions within microbial communities. We propose that our zebrafish infection models will enhance our understanding of microbial interactions and population dynamics and may reveal new approaches to manipulate the microbiome for human health.

Poster #8

CesAB is a type 3 secretion protein chaperone that also protects the mRNA of its protein cargo from degradation to establish a regulatory feedback loop controlling the switch from basal apparatus to translocon filament assembly

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC) is a food-borne pathogen associated with outbreaks of bloody diarrhoea and haemolytic uraemic syndrome in humans. It originates from asymptomatic carriage in cattle and other ruminants. A critical step in EHEC colonisation is attachment and effacement of the intestinal epithelium, dependent on production of a type 3 Secretion System (T3SS) encoded on the Locus of Enterocyte Effacement (LEE). Expression is staged with production of a LEE4 operon-encoded EspADB translocon filament occurring through and onto a membrane-embedded basal apparatus expressed from earlier LEE operons (1-3). SepL is the first protein encoded on LEE4 and is essential for surface production of the EspADB translocon. LEE4 expression (*sepL-espADB*) is tightly controlled at a post-transcriptional level and the transcript's 5' end forms a cloverleaf structure, defined by chemical mapping, that restricts translation of SepL-EspADB. In the present study, using a combination of genetic and protein-RNA binding and degradation assays, we provide evidence that the protein chaperone, CesAB, which is expressed from LEE1 and known to interact with EspA and EspB, also acts on the sepL-espADB transcript to enable translation, primarily by preventing transcript degradation primed by Hfq/sRNA interactions. Once translated the translocon proteins (EspA/B) bind to CesAB countering its positive activity on the transcript, providing an elegant repressive feedback loop. We consider this is the first example of a bacterial protein chaperone also acting on the mRNA of its cargo and highlights the inbuilt regulatory loops inherent in expression, assembly and activity of these complex secretion systems.

Poster #9

Role of a T4BSS in *Yersinia pseudotuberculosis* Far East scarlet-like fever

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Abstract

Yersinia pseudotuberculosis (*Ypstb*) strains responsible for the Far East-scarlet like fever (FESLF) harbor a plasmid encoding a putative type IVB secretion system (T4BSS). By qRT-PCR, we detected *in vitro* an increase in the expression of yT4BSS genes at 37°C compared to environmental temperatures (21°C or 28°C), leading us to hypothesize a role for this system during *in vivo* infections. To address this, we generated a single deletion mutant of the ATPase DotB (essential for T4BSS function in other bacterial species) and we observed a significant attenuation of virulence of the *ΔdotB* mutant when following the survival of orally infected zebrafish larvae or mice. To understand the molecular contribution of the yT4BSS during infection, we analyzed first its potential role for bacterial intracellular trafficking and survival. However, we have not identified differences in the intracellular behavior of WT bacteria versus the *ΔdotB* mutant when applying fluorescence or electron microscopy, nor when performing gentamicin protection assays. To further investigate the functionality of the yT4BSS *in vivo*, we will now compare cytokine profiles in mice infected with WT and *ΔdotB* bacteria to decipher its potential impact on immune functions. Moreover, we have identified *in silico* putative yT4BSS effectors which display molecular features shared with T4BSS effectors of *Legionella pneumophila* and *Coxiella burnetii*: we plan to investigate the translocation of these effectors by the yT4BSS as well as their capacity to interact with host molecules in order to identify signaling cascades that might be hijacked by *Ypstb* during FESLF-related infections.

Poster #10

Type VI Secretion System effector toxicity towards *Pseudomonas aeruginosa*

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Abstract

The type VI secretion system (T6SS) is a contractile nanomachine used by Gram-negative bacteria to deliver toxic effector proteins into adjacent cells. The T6SS of *Vibrio cholerae* has been shown to successfully kill several Gram-negative species. However, *Pseudomonas aeruginosa* is a notable exception in that it is largely unaffected by *V. cholerae* T6SS attacks. Interestingly, *P. aeruginosa* is not inherently resistant to all T6SS attacks, given it is sensitive to attacks from the T6SS of other species, including Acinetobacter baylyi. We endogenously expressed three *V. cholerae* effectors (VgrG3, VasX and TseL) in *P. aeruginosa* to see if *P. aeruginosa* is intrinsically immune to the unique set of effectors delivered by *V. cholerae* T6SS. We found that expression of the effectors in the periplasm but not the cytosol was toxic to *P. aeruginosa*, suggesting that *P. aeruginosa* immunity to *V. cholerae* T6SS may be to the inability of the *V. cholerae* effectors to reach their periplasmic targets. To investigate whether *V. cholerae* effectors are only being delivered to the *P. aeruginosa* cytosol or if the effectors are not able to pass the outer membrane altogether, we developed a fluorescent reporter assay to quantify non-lethal delivery of T6SS substrates to the cytosolic compartment of target cells. In addition to detecting cytosolic delivery of T6SS secretion substrates, this reporter can be used to quantitatively measure what proportion of recipient cells receive T6SS attacks in multispecies bacterial communities.

Poster #11

The *Coxiella burnetii* T4SS effector protein AnkG hijacks the 7SK small nuclear ribonucleoprotein complex for reprogramming host cell transcription

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Abstract

Background: Inhibition of host cell apoptosis is crucial for *C. burnetii* to maintain its intracellular niche and, thus, bacterial survival. The anti-apoptotic activity of *C. burnetii* is mediated by a type IV secretion system (T4SS), which is required to inject effector proteins into the host cell. The T4SS effector protein AnkG is known to exhibit anti-apoptotic properties. How AnkG alters host cell apoptosis is unknown.

Methods: We used different molecular biological methods including Co-immunoprecipitation, immunofluorescence, RNA-immunoprecipitation, RNA-Sequencing, immune-blot analysis and infection experiments with wild-type and AnkG deletion mutant C. burnetii to unravel the mode of action of AnkG.

Results: Here, we report that AnkG binds to the nucleolar RNA helicase 2 (DDX21) as well as to the small regulatory 7SK RNA, which is an important regulator of the positive elongation factor b (pTEFb). AnkG interferes with the function of the 7SK small nuclear ribonucleoprotein (7SK snRNP) complex, leading to significant changes in host cell transcription and ensuring host cell survival. Importantly, AnkG activity is essential for efficient intracellular replication of *C. burnetii* and its ability to inhibit apoptosis.

Conclusions: Our results suggest that the anti-apoptotic effector protein AnkG of *C. burnetii* inhibits host cell apoptosis by altering the activity of the 7SK snRNP complex via releasing p-TEFb and, thus, influencing transcriptional activity. In summary, we identified a novel process by which a bacterial effector protein manipulates the host cell for the benefit of the bacteria.

Poster #12

Alternating rigid and flexible regions of the passenger enhances function of the inverse autotransporter intimin

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Abstract

Inverse autotransporters, or type Ve secretion systems, are adhesins of Gram-negative bacteria exemplified by intimin of enteropathogenic and enterohaemorrhagic *Escherichia coli*. The adhesive extracellular region of intimin (the passenger) is exported through the outer membrane-embedded β-barrel domain through a hairpin-like intermediate. The energy for folding is provided by the sequential folding of individual domains in the passenger. We have solved the crystal structures of the outer-membrane proximal domains D00 and D0, which are both immunoglobulin (Ig)-like domains. However, whereas D0 is structurally very similar to the D1 and D2 domains of intimin, D00 is more divergent. D00-like domains are predicted at the N-terminus of most inverse autotransporters, and recent crystal structures from other representatives of the family confirm this prediction. The connector between D00-D0 forms an S-shaped hydrophobic loop that appears to confer a rigid orientation to the D00-D0 tandem. By contrast, the connections between D0-D1 and D1-D2 are flexible. Making the D00-D0 connector flexible by replacing the residues with glycine and serine did not have an effect on the stability of the domains but had a drastic effect on protein levels in vivo. Simulations show that the rigid connector between D00-D0 increases the reach of the adhesin, whereas the flexibility between D0, D1 and D2 domains allows the distal adhesive D3 domain to adopt the optimal orientation for receptor binding. Thus, alternating rigid and flexible regions within the intimin passenger are important for its function.

Poster #13

Role of the *Campylobacter jejuni* Type VI secretion system and in silico identification of putative T6SS effectors

Janie Liaw[1], Zahra Omole[1], Geunhye Hong[1], Cadi Davies[1], Luca Robinson[2], Dong Xia[3], Nicolae Corcionivoschi[4], Brendan Wren[1], Nick Dorrell[1], Abderrahman Hachani[5], Ozan Gundogdu[1]

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Abstract

Background:

The Type VI Secretion System is a contractile nanomachine that injects effector proteins into bacterial or eukaryotic cells. However, knowledge of the roles of T6SS in *Campylobacter jejuni* and identity of its secreted effectors remain limited despite a high prevalence of T6SS in human and chicken isolates.

Methods:

The whole genome sequence of novel human isolate 488 was compared with previously sequenced strains. Expression of T6SS genes and secretion of TssD were analysed and T6SS mutants were constructed. Oxidative stress assays were performed to determine whether T6SS is associated with the oxidative stress response. Roles of T6SS in interactions with chicken cells were examined using *in vitro* and *in vivo* models. A comprehensive bioinformatic analysis was performed using publicly available genomes to further gain an understanding into genetic architecture of *C. jejuni* T6SS.

Results:

A T6SS cluster was identified to be highly conserved between human and chicken isolates and presence of a functional T6SS was demonstrated. Presence of T6SS increased oxidative stress resistance in *C. jejuni*, indicating T6SS is associated with the oxidative stress response. T6SS enhanced *C. jejuni* interaction and invasion of chicken primary intestinal cells and enhanced ability of *C. jejuni* to colonise chickens. The bioinformatic analysis, in combination with analysis of T6SS secretome, led to identification of a second canonical VgrG and a number of putative effectors.

Conclusion:

These studies highlight the importance of T6SS during in vivo survival of *C. jejuni* and also provide a framework for our current characterisation of *C. jejuni* T6SS effectors.

Poster #14

Characterising putative effectors associated with the *Campylobacter jejuni* Type Six Secretion system

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Abstract

Background: Bioinformatic analysis revealed 19.5% of *C. jejuni* genomes, particularly phylogenetic groups associated with human infection and chicken isolates, harbour Type Six Secretion system (T6SS) operons. Despite this prevalence and previous investigations, no *C. jejuni* T6SS effectors have been formally identified. Our recent bioinformatic analysis, identified *Cj0980* (Tox-REase-7 domain containing protein) and *Cj1003* (metalloprotease) as putative *C. jejuni* T6SS effectors which we aim to characterise.

Methods: Two *Cj0980* and *Cj1003* mutant strains were generated in the T6SS-positive *C. jejuni* strain 488. Co-culture experiments were conducted using T6SS-positive and -negative strains to examine bacterial interactions and possible anti-bacterial properties. Additionally, human intestinal epithelial cells (IECs), T84 and Caco-2, were infected with 488 wild-type and mutant strains to observe influence on host-cell infection.

Results: During co-culture, the *Cj0980* mutant strain no longer displayed a selective advantage against prey T6SS-negative strains. Infection of IEC lines with *Cj0980* mutant resulted in reduced interaction and invasion with respect to 488 wild-type strain, conversely intracellular survival and invasive properties were improved during *Cj1003* mutant infection. Both effector mutants elicited weaker induction of the chemokine IL-8 after infection; a phenotype which has been observed in *C. jejuni* strains unable to adhere and invade IECs.

Conclusion: Mutation of *Cj0980* abolished co-culture competitive advantages observed with 488 wild-type and *Cj1003* mutant strains suggesting a role for *Cj0980* in intrastrain antagonism. *Cj1003* mutant strain assays identified interesting phenotypes proposing a function in IEC infection. These preliminary findings warrant further investigation to help fully elucidate the function(s) of these putative effectors.

Poster #15

Insights into the structural diversity of mycobacterial type VII secretion systems and mechanisms of protein translocation

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Abstract

The type VII secretion system is required by mycobacteria for the translocation of a range of substrates, that are key for a diverse set of functions including nutrient uptake, DNA transfer and immune modulation. Slow growing, often pathogenic, mycobacteria encode up to five of these systems (ESX-1 to ESX-5). Our recent work solved the high-resolution structure of the ESX-5 system from *Mycobacterium xenopi* using a combination of cryo-EM, X-ray crystallography and crosslinking mass spectrometry studies (1). The resulting model along with other structures of the ESX-5 and ESX-3 systems and improved structure prediction methods have paved the way for structural comparison of the different systems.

In addition, further structural analysis of the M. xenopi ESX-5 pore complex highlighted residues that we hypothesised would play a role in mediating secretion. We probed the role of these residues by generating several site directed mutants and tested the impact of these residues on the structure and function of the ESX-5 complex. Based on this data, we will present our recent insights into the secretion mechanism of the ESX systems and how their structural diversity may mediate their substrate specificity.

1. K. S. H. Beckham, C. Ritter, G. Chojnowski, D. S. Ziemianowicz, E. Mullapudi, M. Rettel, M. M. Savitski, S. A. Mortensen, J. Kosinski, M. Wilmanns, Structure of the mycobacterial ESX-5 type VII secretion system pore complex. Sci. Adv. 7 (2021)

Poster #16

Characterisation of the *Enterobacter bugandensis* type VI secretion systems

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Abstract

The type VI secretion system (T6SS) is a transmembrane nanomachine used by Gram-negative bacteria to antagonise and inject effectors into neighbouring cells. Although it primarily functions in bacterial competition, a number of studies demonstrate a role in pathogenesis. Unpublished findings from our lab show that the T6SS is involved in *E. bugandensis* virulence. *E. bugandensis* is a member of the ESKAPE pathogens and is of growing concern due to its hyper-virulence and high levels of multi-drug resistance. Therefore, the aim of this study is to investigate the role of the *E. bugandensis* T6SS in pathogenesis and bacterial competition. To characterise the T6SS and identify potential effector candidates for further study, we analysed the genome *in silico* using a variety of bioinformatic tools and the secretome *in vitro* via mass spectrometry. The *E. bugandensis* E104107 genome contains two T6SS gene clusters, termed T6SS-1 and T6SS-2. T6SS-1 contains all 13 core components required for secretion, whereas T6SS-2 lacks several of these core components. E104107 possesses 9 *hcp* genes scattered across the genome, as well as 3 *vgrG* genes: two within T6SS-1 (*vgrG2* and *vgrG3*) and one outside the cluster (*vgrG1*). *vgrG1* and *vgrG2* possess additional c-terminal domains which may represent specialised effectors. Ten other potential cargo effectors were identified, including 2 unknown PAAR domain-containing genes. Furthermore, some candidates identified have no known sequence or structural homology to other proteins and may represent novel effectors. Further studies are ongoing to investigate the role of these effector candidates in pathogenesis and bacterial competition.

Poster #18

Type VII Secretion System Substrates mediate Vitamin B12 Transport in Mycobacteria

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Abstract

Vitamin B12 (B12) is a complex molecule that participates as a cofactor in important metabolic pathways. Mycobacteria present three enzymes that depend on this vitamin. One of these is MetH, a methionine synthase that catalyzes the conversion of L-homocysteine into L-methionine.Moreover, B12 can also regulate gene expression through a riboswitch. This riboswitch inhibits transcription of *metE* that encodes for an alternative methionine synthase. We explored the use of these enzymes to identify B12 transport proteins in mycobacteria.

We constructed a *metH* mutant, which was sensitive to B12 and demonstrated that *Mycobacterium marinum*, a commonly used surrogate of *Mycobacterium tuberculosis*, can use exogenous B12. Vitamin B12 is a bulky and water-soluble molecule, thus we speculated that outer membrane proteins could mediate its transport. Interestingly, mycobacteria present a second B12-sensitive riboswitch located upstream of a *ppe* gene. PPE proteins are substrate of the Type VII secretion family and some are membrane proteins involved in nutrient uptake. We then created a mutant of the *ppe* gene in the *metH* mutant background, which was resistant to B12. Cell fractionation experiments showed that PPE was soluble and not detectable in the envelope, suggesting a different role on B12 uptake. To identify other proteins required for B12 transport, we selected mutants that were resistant to exogenous B12 in the *metH* mutant strain. The analysis showed that other type VII substrates were responsible for this process. In summary, our data indicate that vitamin B12 can be scavenged by *M.marinum* and this transport is dependent on substrates of the Type VII secretion system.

Poster #19

The C-terminal targeting sequence of autotransporter Hbp is needed to initiate Bam complex-mediated secretion across the outer membrane.

Peter van Ulsen

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Abstract

The classical monomeric autotransporters, a major subclass of the Type V secretion systems, are ubiquitous in Gram-negative bacteria and function in virulence and colonization. They are also considered attractive tools to expose proteins of choice at the cell surface. Autotransporters pass the inner and outer membrane (OM) in two consecutive steps facilitated by the Sec translocon and the Bam complex, respectively. The latter is the insertase for integral β-barrel proteins into the OM. It is well-established that this complex is required for autotransporter secretion and autotransporters include a β-barrel at their C-terminus. The targeting of the Bam complex is proposed to involve the last β-strand of the β-barrel. I investigated this using the autotransporter Hbp of Escherichia coli as model and show that deletion of its C-terminal phenylalanine residue blocks secretion, but does not hamper the ability to fold into a β-barrel. By contrast, mutating the last three residues both blocked secretion and prevented barrel formation. These results suggest a different interaction with the Bam complex, which is further corroborated by the sensitivity of the constructs to periplasmic protease DegP.

Poster #20

Unraveling the Type IV-like Secretion System of Conjugative Plasmids in Mycobacteria

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Abstract

The most widespread mechanism for plasmid conjugation depends on a type IV secretion system (T4SS). Recently, conjugative plasmids were discovered in mycobacteria. An unusual characteristic of these plasmids is that they depend on the presence of both a type IV-like and a type VII secretion system (T7SS) for conjugation. These conjugative plasmids seem to be widespread among mycobacteria and have been suggested to play an important role in the divergence of T7SSs. In this study we analyzed the type IV-like secretion system of plasmid pRAW of M.marinum E11.
This type IV-like secretion locus consists of 13 conserved genes of which only three are homologous to known T4SS components, namely VirB4, VirD4 and VirB8. Most of the other conserved genes in this locus have no homologues outside these plasmids. We generated mutations in these genes using a CRISPR-Cas9 directed mutagenesis approach to determine their involvement in conjugation. We show that most of these genes are indeed essential for plasmid conjugation, suggesting they might have roles equivalent to the absent T4SS components and form a molecular machine required for DNA transport. We have generated AlphaFold predictions for singular proteins and various combinations. Currently, we are testing the putative interactions that we have identified. We are also investigating how the type IV-like secretion system interacts with the T7SS and its substrates. Understanding this conjugative plasmid that requires two secretion machineries will provide more insight into the exchange of DNA between slow-growing mycobacteria and its contribution to their evolution.

Poster #21

Use of SIMPLEx for studying proteins of the type II secretion system

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Abstract

The type II secretion system (T2SS) is harnessed by gram-negative bacteria to elicit disease. Although many of the structures of individual components or their domains have been resolved, the inner membrane platform is still elusive. There are many unresolved questions about the mechanism of this secretion system: importantly the role of the assembly platform in pilus biogenesis and substrate secretion across the outer membrane. GspF is the potential rotor protein responsible for assembling the pilin subunits into the short pilus, but its full-length structure is unknown. We have successfully expressed and purified GspF homologues using the SIMPLEx system to homogeneity and optimised the structural target for high-resolution CryoEM single particle analysis. Samples are vitrified on holey Quantifoil Au grids (R 1.2/1.3, 300 mesh) using the Leica Cryo plunger. A high-resolution dataset of 3677 movies at a nominal magnification of 81000 corresponding to 1.1 Å/px and a defocus range of -0.7 to -3 µm and total electron dose of 50e/Å2/s has been acquired using LonCEM Titan Krios and data processing is underway using RELION-4.0-beta. 1,700,000 particles were automatically picked by crYOLO after model training and used for 2D classification. After three rounds of 2D classification particles were used for initial model building and 3D classification to obtain a homogeneous particle dataset for further refinement. Current lower resolution models (~12Å Gold-standard FSC) reveal a dimer-like architecture with overall dimensions of ~70Å x 62Å x40Å. Progress with the structure will be presented at the meeting.

Poster #22

Investigating the role of the staphylococcal type VII secretion system in cell surface integrity

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Abstract

Staphylococcus aureus is a successful human pathogen with a notable ability to acquire resistance to many antibiotics. *S. aureus* encodes a specialised type VII secretion system (T7SS), a key virulence factor, which is considered to be a good drug target. However, the role of the T7SS in bacterial physiology and infection remains unclear. We recently reported that T7SS mutants are more sensitive to fatty acids, which was attributed to altered cell membrane integrity. Here we demonstrate that *S. aureus* strains lacking T7SS effectors EsxC and EsxA show an altered cell surface morphology using scanning electron microscopy. LC/MS analysis showed altered protein profiles of membrane preparations from the wild type (WT) and mutants, and the localisation of cell membrane proteins like flotillin were altered in the mutants. Additionally, a 'less positive' whole cell surface charge was measured in *esxC* and *esxA* mutants using a quantitative binding assay. To investigate if the surface changes would impact bacterial sensitivity to membrane targeting antibiotics, we studied effects of the last resort drug, daptomycin, on T7SS mutants. We found that T7SS mutants display defective growth *in vitro* in the presence of daptomycin, compared to the WT. Microscopic analysis of propidium iodide stained, daptomycin-treated bacteria showed an increased cell death in the mutants. Furthermore, the intracellular survival of T7SS mutants in cells treated with daptomycin was lower than the WT. Thus, the staphylococcal T7SS may contribute to the maintenance of cell surface integrity and as a result affect sensitivity to membrane-acting drugs such as daptomycin.

Poster #23

Revealing membrane insertion mechanisms of Legionella's integral membrane effector proteins in host cells

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Abstract

A virulence strategy used by the intracellular pathogen Legionella pneumophila is to manipulate host cellular processes to survive within phagocytic host cells. Hence, more than 300 virulent effector proteins are delivered into the host cells by the specialized Dot/Icm type IV secretion system (T4SS). Many effector proteins harbor hydrophobic transmembrane-domains (TMDs) to fulfill their function in host cell membranes. However, the mechanisms T4-secreted TMD-effectors (TMEs) use to target and insert into the correct membranes of eukaryotic hosts still need to be unraveled.
Focus will be on a small selection of L. pneumophila TMEs, which all possess diverse properties regarding their size, hydrophobicity or number of TMDs to dissect intrinsic features decisive for particular membrane targeting and insertion mechanisms. To understand the relevance of host cell targeting factors and receptors involved in membrane insertion of bacterial TMEs, we will compare their localization after T4SS-facilitated injection or in-host ribosomal translation by live-cell fluorescence microscopy or subcellular fractionation. To co-localize TMEs with distinct membrane compartments a protocol for the subcellular fractionation of eukaryotic cells was established and optimized. Macrophages were lysed by glass bead milling, and a precise separation of organelles could be achieved by combining differential centrifugation with subsequent sucrose density gradient centrifugation. Moreover, we will investigate TME interactions with host proteins by proximity biotinylation or photocrosslinking, followed by mass spectrometry-based protein correlation profiling to reveal their targeting and insertion pathways.

Poster #24

Core and accessory effectors of type VI secretion systems contribute differently to the intraspecific diversity of *Pseudomonas aeruginosa*

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Abstract

The function of type VI secretion systems (T6SSs) depends on their effector proteins. Little is known about the effectors' diversity within a species. Here, we perform comparative genomics to systematically test for the intraspecific diversity of T6SS effectors in phylogenetically distinct *Pseudomonas aeruginosa* strains. We found core effectors, which are omnipresent and conserved across the species, and accessory effectors, which vary between strains by presence-absence or in their enzymatic activity. Each of the three T6SSs of P. aeruginosa translocates at least one core effector. Accessory effectors are associated to the T6SSs at their tip through PAAR- or VgrG-domains but not through Hcp proteins, while core effectors can also be found in the inner tube. The combination of core effectors with various accessory effectors results in an effector set that can be specific to a particular strain. Out of 960 possible effector sets of the observed effector variants, we find 36 distinct sets in a local population of 52 diverse strains. Some effector sets are found in one strain only. Other sets are more abundant and might provide a broad benefit. Most of the analyzed strains differ in their effector set from studied laboratory reference strains and call for more research on diverse isolates to understand the role of the T6SS for *P. aeruginosa.*

Poster #25

Assembly, structure and function of mycobacterial type VII secretion systems

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Abstract

Mycobacterium tuberculosis causes one of the most important infectious diseases in humans, leading to 1.4 million deaths every year. Specialized protein transport systems, called type VII secretion systems (T7SSs), are central for its virulence, but also crucial for nutrient and metabolite transport across the specific and diderm mycobacterial cell envelope. Here, we use reconstituted ESX-5 systems in the avirulent species *Mycobacterium smegmatis*, that lacks ESX-5, to get structural and functional insight into the T7SS machinery. Using these, we solved the structure of an intact T7SS inner membrane complex: a 2.32 MDa ESX-5 assembly, consisting of five conserved membrane components and 165 transmembrane helices. By solving several structural subpopulations of this nanomachinery, we highlighted the role of the MycP protease in stabilizing the entire machinery and we determined two different conformations for the large EccC ATPase. Subsequently, we defined the role of each *esx-5* gene in functionality, expression and assembly of the membrane complex by creating a complete array of gene deletions. This allowed us to propose a model of assembly and multimerization of the inner membrane complex. Moreover, we show that the Esx and PE/PPE heterodimeric substrates are dependent on each other for secretion, with both PPE substrates required for secretion and localizing to the cell envelope. Based on this, we hypothesize that specific PPE proteins, encoded by the ESX systems, function as outer membrane channels that mediate protein transport across this specific second membrane in mycobacteria.

Poster #26

Biogenesis of Peptidoglycan and Lipopolysaccharide around Bacterial Protein Secretion Systems

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Abstract

The cell envelope serves as the front-line to both defence and pathogenicity in bacteria. Critical protein assemblies are required to mature, localise and assemble proteins, oligosaccharides and lipids around the cell to enable protection against antibiotics, phages and toxins. Furthermore, these components establish the protein secretion systems in place to allow interactions at the host-pathogen interface. Here we will describe structures and dynamics of membrane protein systems responsible for the maturation of the peptidoglycan cell wall and outer membrane of Gram-negative bacteria. We uncover the molecular basis of how two glycosyltransferase enzymes, RodA and WaaL engage with their substrates to allow production of, respectively peptidoglycan and lipopolysaccharide. We show that both enzymes share a common protein fold, have similar binding sites for their substrates, and have comparable metal-independent catalytic mechanisms for the production of their products. We use molecular dynamics simulations to dynamically dock the Lipid II substrates to RodA, revealing binding sites, either side of the protein, for two Lipid IIs to bind. We show that both cavities are preserved in WaaL, for the binding of lipid-linked O-antigen and Lipid A core OS, the precursor to mature Lipopolysaccharide. Substrates in both cases are coordinated by arginine residues, positioning the ligands in apposition of a catalytic aspartate (RodA) or histidine (WaaL), and enabling a catalytic mechanism to be proposed. We hypothesise that the commonalities between these two critical enzymes and their role in assembling the bacterial cell envelop offers the potential for developing novel antimicrobial inhibitors for both glycosyltransferase processes.

Poster #27

Understanding the assembly mechanism of the Twin Arginine transport system

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Abstract

The twin arginine transport (Tat) system transports folded proteins across the prokaryotic cytoplasmic membrane and the plant thylakoid membrane. The E. coli Tat system consists of three integral membrane proteins, TatA, TatB and TatC. Unlike conventional protein translocation pathways that contain an aqueous transmembrane channel through it the substrate can migrate, Tat transporter seems to work by a nonconventional mechanism in which a translocation conduit is assembled on demand. Substrate binding promotes a conformation rearrangement of the Tat complex and the recruitment of TatA to the Substrate-TatBC complex, where it polymerizes to form the substrate translocation pathway. The mechanism by which folded proteins are transported across the membrane is still poorly understood. In this study, we used coarse-grained MD simulations to describe the TatA oligomerization mechanism and to understand how the translocation pathway is formed. We demonstrated a new potential TatA binding site located at TatC TM1, besides the previously reported TM5/TM6 binding sites. Our data show that the Tat complex becomes more dynamic after substrate binding. The presence of the substrate induces a “breathing” movement, increasing the separation between two TatC subunits. Moreover, substrate binding promotes an asymmetry in the Tat Complex, which favours the pore formation at the TatC-TatC interface opposite to the signal peptide binding site, with the TM6 and TM1 acting as a support for the TatA oligomer. Our extensive CG simulations provides a molecular description for assembly of the active Tat translocase and pore formation.

Poster #28

Adaptive immune response against a type VI secretion system (T6SS) protein in a cystic fibrosis patient colonized with *Pseudomonas aeruginosa*

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Abstract

Antibodies against T6SS proteins were detected in the blood of cystic fibrosis patients, indicating that the T6SS is a target of adaptive immunity during chronic lung infections. However, the adaptive immune response against the T6SSs of *P. aeruginosa* and its potential role in pathogenesis is poorly understood. Here, we (i) characterize the T6SSs of *P. aeruginosa* isolates and (ii) determine the adaptive immune response to the T6SS of *P. aeruginosa* in one and the same patient to gain a better mechanistic understanding of T6SS-mediated immune activation.

P. aeruginosa colonies were directly isolated from the patient's sputum sample. The isolates were confirmed by PCR using species-specific primers, and subjected to whole genome sequencing. All of the isolates harbor *hcp1* in their genomes, which encodes one of the most abundant structural proteins of the H1-T6SS. One of the isolates expresses the protein under laboratory conditions, demonstrating phenotypic diversity among the isolates.

An ELISA was designed to determine the titer of anti-Hcp1 antibodies in the patient. We observed a robust and specific response against Hcp1. This response is considerably higher compared to the response against two cytoplasmatic *P. aeruginosa* proteins. These findings support previous data and highlight the potential of Hcp1 being a particularly potent trigger of the immune system.

Poster #29

Insertion mechanism of *Salmonella*`s integral membrane effector proteins SseF and SseG into host cell membranes

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Abstract

Salmonella Typhimurium invades and replicates inside eukaryotic cells by injecting virulent effector proteins via two versions of the type III secretion system (T3SS) into its host. While T3SS-1 is important for invasion, the T3SS-2 is necessary for the survival of the bacteria inside the eukaryotic cell. Among the T3SS-2 injected effectors are the integral membrane proteins SseF and SseG (SseFG). After their injection into the hosts cytosol, it was shown that SseFG are located in the membrane of the *Salmonella*-containing vacuole. However, the mechanism behind the insertion of SseFG into the host cell membranes is not known.

To address this question, the localization of SseFG after T3SS-2 injection vs. in-host-translation will be compared using different approaches: fluorescent live cell microscopy, subcellular fractionation, and proteomics. First, the protocol for subcellular fractionation of the host cell organelles (i.e., plasma membrane, endoplasmic reticulum, lysosome, golgi and mitochondria) was optimized. Here, HeLa cells were homogenized by bead mill for 30 seconds, followed by two differential centrifugation steps and the separation of the two pellets (P1 and P2) by a sucrose gradient (30-60 % w/w). After fractionation of the sucrose gradient of P1, the cell organelles were detected in each of the twelve fractions. However, each cell organelle seemed to be more enriched in a different fraction. In P2, only lysosome, plasma membrane and mitochondria were detected. All in all, an additional mass spectrometry analysis is required to evaluate the actual number of cell organelles in the different fractions.

Poster #30

Type VII Secretion System Effectors of *Staphylococcus aureus* at The Host Pathogen Interface.

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Abstract

Staphylococcus aureus, a major hospital and community associated pathogen, causes infections ranging from impetigo to bone infections, which are attributed to the wide range of virulence factors it possesses. One such virulence-associated system is the type VII secretion system (T7SS), which exports several effectors including EsxA and EsxD. EsxA has been implicated in modulating host epithelial cell apoptosis during intracellular infections, although the underlying mechanisms are unclear. To study how EsxA interacts directly with host factors, the mammalian protein interaction partners of EsxA in epithelial cells were explored using different protein interaction screens, including a proximity-based biotin identification method (BioID). The purified interacting proteins were identified using liquid chromatography mass spectrometry (LC-MS). A number of host proteins involved in cellular trafficking and cell death pathways were identified, which are being characterised. To understand the function of T7SS effectors during infection in vivo, deletion mutants lacking specific effectors were studied in a intradermal murine skin infection model. While mutants lacking EsxD did not show any significant differences in the bacterial load in the skin and distal organs, we observed differences in the progression of the skin abscess over the 3-4 days of infection. We are currently investigating this further through flow cytometry, chemokine and cytokine profiling of the infected abscess. Thus, our data demonstrate an important role of T7SS during infection and may inform development of better vaccines for *S aureus* infections.

Poster #31

Type VI Secretion System (T6SS) as weapon in biocontrol of phytopathogenic bacteria

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Abstract

T6SS are contractile nanomachines used to propels effectors directly into targeted bacteria and eukaryotic cells or the nearby extracellular environment through an assembly/contraction mechanism.

The main role described for these systems is antibacterial activity. Indeed, by delivering toxins against targeted bacteria, T6SS provides a competitive advantage in a given environment compared to strains lacking T6SS. These T6SSs are often involved in virulence mechanisms due to pathogens like *Vibrio cholerae* or *Pseudomonas aeruginosa* towards human cells in which they inflict major damages. However, some T6SSs are also involved in beneficial processes, especially in the rhizosphere, where strains with T6SS can exert a biocontrol activity.

The *Pseudomonas fluorescens* strain MFE01 deploys its T6SS to protect plants against the *Pectobacterium atrosepticum* phytopathogen, by causing a reduction in pathogen cell density. This activity was previously evaluated both in vitro and in planta by competition assays (Decoin *et al*., 2014). The construction of a translational fusion of the T6SS (*tssB*::*sfgfp*) allowed to visualize and dissect the bactericidal scenario leading to the pathogen cell rounding prior to lysis. Effector responsible for this rounding is under investigated by biochemical and microscopic approaches. In addition, cell observations with super-resolution microscopy (3D SIM) highlighted amazing traits carried by this strain such as distribution of T6SSs on both sides of the cell including in various axes. *P. fluorescens* strain MFE01, through it T6SS, could be a good biocontrol strategy to fight against phytopathogenic bacteria and thus be a good alternative to reduce the use of chemicals.

Poster #32

Secretion Mechanism of Burkholderia Lethal Factor 1 by Outer Membrane Vesicles

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Abstract

Burkholderia lethal factor 1 (BLF1) is an important virulence factor produced by Burkholderia pseudomallei, the causative agent of melioidosis, and the first B. pseudomallei toxin with role in virulence identified. Recombinant BLF1 is toxic for macrophages and epithelial cells, and kills mice challenged by the intraperitoneal route. BLF1 is a glutamine deamidase that specifically deamidates Glu339 in eIF4A (eukaryotic initiation factor 4A). This modification inactivates the RNA helicase activity required for melting mRNA secondary structures during initiation of translation, which this leads to extensive inhibition of protein synthesis in human cells. How this toxin is translocated from the bacterial cytoplasm to the surrounding environment is unknown. Our data suggest that BLF1 is a secreted protein that can be detected in culture supernatants and its secretion and translocation into macrophages is not related to any secretion system (T2SS, T3SS or T6SS). We found a region in the protein that is critical for its secretion and transport to the periplasm. Furthermore, we discovered that secreted BLF1 was associated to outer membrane vesicles (OMVs). The toxin is likely located at the surface of the OMVs since it is accessible to digestion by proteinase K. Together, we conclude that BLF1 is secreted to the extracellular milieu via OMVs and does not utilise any of the classical secretory pathways.

Poster #33

Functional and evolutionary analyses of Xanthomonas translucens Type VI Secretion System

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Abstract

The phyllosphere is the site of dynamic and complex polymicrobial interactions, indirectly impacting the host plant's health. While bacteria of the genus Xanthomonas are considered major phytopathogens, type I to VI secretion systems (T1SS-T6SS) have been identified among the different Xanthomonas species. Among these, the T3SS is the most studied for its role in bacterial pathogenesis. T1SS, T2SS and T5SS seem mainly responsible for adhesion and degradation of host cell wall, while T4SS is involved in conjugation and bacterial killing. In addition, to compete with nearby microorganisms, many bacteria deploy T6SS to inject toxic effectors into prokaryotic or eukaryotic target cells. Previous work revealed the presence of three distinct T6SS clades within the Xanthomonas genus, yet our current understanding of their function and the adaptation events leading to the gain or loss of these clusters still remains limited. Here, a genomics-informed screening for T6SS gene clusters was conducted among all clade-1 xanthomonads. We took advantage of the patchy distribution of T6SS clades within the cereal pathogen Xanthomonas translucens to address the question of T6SS's biological functions. Using knockout and fluorescence approaches, we showed that one T6SS is required for inter-bacterial competition, while no evidence for a role in virulence on barley was found. Using a combination of comparative genetics and phylogenetic analyses, we described the evolutionary features of T6SS clusters along the X. translucens phylogeny. Functional and evolutionary analyses of X. translucens T6SS provide new insights into the mechanisms promoting early infection steps at cereal leaves surface.

Poster #34

Using the power of cryo-EM to elucidate the molecular mechanism of bacterial competition in *Clostridium difficile.*

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Abstract

Many bacterial species exist in complex communities, whereby fierce competition occurs to exhaust microbial niches, for example via toxin warfare. *B. subtilis* utilises an antimicrobial peptide, SDP, in an unusual mechanism of lifestyle switching or 'cannibalism' as a last resort before commitment to the energetically costly process of spore formation. Interestingly, this pathway may present a promising target for the development of novel antimicrobial therapies, as evidence suggests it may be implicated in interbacterial competition. In this obscure process, a subset of the population releases SDP, which is expressed in an operon alongside two proteins; SdpA and SdpB are necessary for the cleavage and activation of the precursor protein, SdpC, yet their modes of action are unknown. Furthermore, a non-canonical ABC transporter, Ykn, affords the attacking cells with additional protection against SDP in B. subtilis and Ykn homologues are found in C. difficile. Conditions for the over-production and purification of mature SDP and SdpABC were identified herein, a necessary precursory step for structural characterisation. We aim to further understand this cannibalism mechanism using an integrative approach, which combines structural biology techniques with functional knockout studies in *C. difficile.* Specifically, cryo-EM will be applied to resolve the first structures of Ykn and SdpABC, with potential to solve the structure of SdpA first by X-ray crystallography. NMR will be exploited to elucidate the structure of the peptide toxin, SDP, alongside biophysical characterisation.

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