Instituto Juan March
de Estudios e Investigaciones

157 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Finding the Way Out: Protein Traffic in Bacteria

Organized by

A. P. Pugsley and V. de Lorenzo

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Introduction

Anthony P. Pugsley and Víctor de Lorenzo

Bacteria manage their limited amount of genetic information in an exquisite manner, exploiting all imaginable devices at the molecular, physiological and cellular levels to survive and proliferate. The containment and compartmentalisation that were essential requirements for the development of life are incompatible with the need to derive nutrient from and communicate with the environment. Containment and compartmentalisation are achieved through the creation of biological membranes based on lipids, hydrophobic molecules that form an essentially impermeable barrier between the cell (or organelle) and its surroundings. This permeability barrier is rendered selectively permeable by the introduction of proteins and protein complexes that form channels and energy-dependent transporters that permit or promote the movement of ions, solutes and even macromolecules across the membrane.

In eukaryotic cells, protein traffic occurs at several levels (into and out of organelles and out of the cell) and by a variety of different mechanisms. Although most prokaryotes do not have internal organelles, they are nevertheless capable of specifically and efficiently localising proteins to different sites, and by a variety of different mechanisms of varying degrees of complexity. Indeed, the number of different protein traffic pathways in bacteria is surprisingly high, reflecting their different origins, parallel evolution and adaptation to specific substrate proteins or to specific functions. For example, bacteria are not only capable of assembling proteins in their cell envelopes but can also assemble protein appendages or coats on their surface (pili, flagella and S-layers), can secrete proteins into the growth medium and can inject proteins into target cells that they infect. Considerable progress has been made over the past ten years in the molecular dissection of these protein traffic mechanisms. While many of them are specific to bacteria but others share many features with major protein traffic pathways in eukaryotes and, in all cases, the study of bacterial protein traffic has revealed invaluable insight into the problems encountered when moving a protein from one side of a membrane to another.

Broadly speaking, protein traffic can be divided into two major processes, depending on whether or not the protein that is transported is in an unfolded or folded configuration before and during its transport through the membrane. Surprisingly, however, the degree of complexity of the transport machine does not always seem to correlate with the extent to which the protein that is transported is folded. Furthermore, the distinction between the two is not always clear because there is no practical way of determining the conformation of a protein in situ before it is translocated. However, it is relatively well accepted that proteins that are translocated in an unfolded configuration must be maintained in a translocation competent state, presumably by chaperones with anti-folding activity, prior to translocation, or be translocated in a co-translational manner. In these cases, the proteinaceous channel through which the protein is translocated will have a relatively narrow diameter. In contrast, proteins that are folded prior to translocation might need chaperones to assist their folding and/or to

verify that folding has occurred before translocation is permitted, and the channel through which the proteins are transported must have relatively large diameter. In both cases, energy is required to achieve translocation.

Recent structural analyses of membrane protein complexes involved in bacterial protein traffic are beginning to reveal fascinating insights into the molecular mechanisms of protein translocation in bacteria. Supramolecular structures can be visualised by electronmicroscopy, which reveals information on the overall dimensions of the machines and their potential capacity to handle folded or unfolded proteins. Higher resolution X-ray crystallographic analysis reveals atomic level details of the structure of these machines in which putative translocation channels can be identified. However, these structures do not reveal all that is to be learnt. Instead, they provide a fixed framework on which to design experiments to determine how they function.

Finally, the growing interest in understanding how secretion works is not altogether devoid of practical implications: protein export systems *à la carte* can be developed today not only as cell factories for secreting high-added value polypeptides into the external culture medium. They can also be instrumental for delivering active proteins to the niches where such microorganisms may naturally reside (the animal gut, the skin, polluted soil, the rhizosphere) for protection against pathogens, for remediation of chemical contamination or biological control of pests. Finally, some secreted proteins are at the basis of the virulence of many bacterial pathogens. These features open up new possibilities of screening novel drugs and compounds specifically able to inhibit given secretory mechanisms.

Whether basic or applicable, all these challenges in protein secretion will keep us busy for the next few years!

Anthony P. Pugsley
Victor de Lorenzo

Session I
Chair: Shin-Ichi Aizawa

Bacterial protein translocase molecular mechanism

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Protein export is a unique protein folding reaction that is temporally delayed and topologically guided. Specialized secretion pathway chaperones recognize nascent secretory chains and guide them to the plasma membrane. Subsequent protein secretion and membrane protein integration occurs through the Sec preprotein translocase. Translocase is built of a transmembrane pore or clamp (SecYEG proteins) thought to form a "channel-like" structure and a peripheral motor (SecA) that binds to the channel. SecA is a DEAD motor helicase and uses ATP-derived, energy to undergo cyclic changes of its conformation. During these cycles significant portions of SecA enter the membrane plane, transfer and release preprotein segments and then regress. These changes allow the translocase machine to move processively along hundreds of preprotein substrates destined for export with defined "steps" of 20-40 aminoacyl residues. At the core of SecA lies an aminoterminal domain that is structurally homologous to those of other *bona fide* nucleic acid helicases and was henced termed "DEAD motor". The DEAD motor is built of two nearly identical subdomains: NBD (nucleotide Binding Domain) and IRA2 (Intramolecular regulator of ATPase) that form between them a nucleotide binding cleft for a single ATP molecule. Two add-on domains that provide SecA with substrate specificities sprout out of each of the primary DEAD motor subdomains: SSD (substrate specificity domain) is rooted in NBD, while the C-domain is rooted in IRA2. The C-domain has four distinct subregions (Scaffold Domain, Wing Domain, IRA1 and the extreme C-terminal redion) and binds to the DEAD motor. IRA1, a helix-loop-helix structure is of particular importance in SecA structure and catalytic function. IRA1 performs two essential tasks : a. it is a global regulator of all SecA catalytic activities and ligand interactions and b. it controls conformational cross-talk between the DEAD motor and the specificity domains SSD and C-domain. Thus IRA1 co-ordinates and couples the ATP-driven conformational cycles of SecA to SecYEG and preprotein binding and release cycles. A model that attempts a synthesis of the available biochemical and biophysical data will be discussed.

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Bypassing the periplasm: the mechanism underlying bacterial type I protein export and multidrug efflux

Vassilis Koronakis

The type I pathway of Gram-negative bacteria is central to protein export and drug efflux. Analysis of the export of E.coli hemolysin, revealed reversible substrate-induced assembly of a contiguous trans-periplasmic channel comprising a two component inner membrane translocase and the outer membrane protein TolC. Recent dissection of the opening and closing of the TolC channel-tunnel will be described, and its interaction with the inner membrane tranlocases that direct toxin export (HlyBD) and multidrug efflux (AcrAB, EmrAB).

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Structure, folding and membrane insertion of the outer membrane protein OmpA

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The outer membrane protein A (OmpA) has served as an excellent model for studies of folding and membrane insertion of outer membrane proteins. Although OmpA serves predominantly a structural function, it also functions as phage and colicin receptor, is involved in bacterial conjugation, and exhibits ion channel properties when reconstituted into planar membranes. We have recently determined the structure of the 19 kDa transmembrane domain of OmpA in detergent micelles by multidimensional hetero-nuclear solution NMR. The backbone fold is characterized by a rigid 8-stranded β-barrel structure with long flexible loops extending from the extracellular side. Measurements of backbone dynamics by NMR reveal a few charged luminal residues with elevated conformational dynamics, which likely reflect their involvement in the gating of the ion channel.

Kinetic folding studies reveal a detailed concerted mechanism of folding and membrane insertion of OmpA. Several membrane-bound kinetic folding intermediates can be distinguished. Although the first membrane-binding step probably involves some secondary structure formation, most secondary and tertiary structures are formed synchronously in subsequent folding and insertion steps. The kinetics of folding and insertion depend strongly on the lipid species in the membrane, with much faster rates in thin than in thick lipid bilayer membranes. Pure phosphatidylethanolamine bilayers do not support folding and insertion of OmpA, but the chaperone Skp and lipopoly-saccharide may assist this process. A folding system has been developed to measure equilibrium folding/unfolding of OmpA in membranes. Systematic variations of lipid compositions show that the thermodynamic stability of OmpA, and by extension presumably also of most other integral membrane proteins, is strongly modulated by elastic material properties of the lipid bilayer.

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Surface proteins of *Listeria monocytogenes*

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Listeria monocytogenes is a gram positive bacterium which has emerged as model system to study host pathogen interactions, especially those of intracellular organisms. Indeed, *L. monocytogenes* has many interesting properties which allow to address various aspects of its life style and in addition its orginal property to trigger its own entry into cells which are normally non phagocytic.

Our laboratory has a long lasting interest in the study of proteins which are displayed on the listerial surface and appear as the primary tools used by the bacterium to interact with the mammalian cell. Among them, internalin which allows entry into epithelial cells, InlB which also permits entry into various cell types, ActA which induces actin polymerisation to only cite the best characterized ones.

The recent elucidation of the genome sequence has allowed to identify that the bacterium has an exceptionally large repertoire of surface proteins. They belong to several categories of surface proteins which will be discussed during the talk. Our primary goal is to identify which of them play a critical role in virulence. Several approaches have been taken, including the comparison of *L. monocytogenes*(strain EGD) surface proteins to that of *L. innocua*, a non pathogenic *Listeria*, and to that of several *L. monocytogenes* strains, as well as the inactivation of two sortases.

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Genetic dissection of lipoprotein sorting within the envelope of Gram-negative bacteria

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Lipoproteins represent a numerically minor but nevertheless important family of exported proteins in bacteria, characterised by the presence at their N-terminal extremity of (up to) three fatty acyl chains. The Sec machinery mediates their export across the cytoplasmic membrane. In Gram-positive bacteria, lipoproteins remain anchored in the membrane by their fatty acids. In Gram-negative bacteria, lipoproteins are found in three locations: in the cytoplasmic membrane facing the periplasm, in the outer membrane and facing the periplasm and in the outer membrane facing the outside of the cell. In most cases, lipoproteins are devoid of other membrane anchors (transmembrane alpha helices for cytoplasmic membrane proteins or amphipathic beta strains for outer membrane lipoproteins). In some cases, proteolysis close to the lipid anchor can lead to the release of the bulk of the polypeptide into the surrounding medium.

In Gram-negative bacteria, the main sorting pathway for outer membrane lipoprotein localisation is the Lol pathway, discovered and extensively characterised by H. Tokuda and his colleagues. Interaction with the Lol system is believed to determined by the presence or absence of an aspartate residue at position +2 of the fatty acylated, mature lipoprotein. We are using genetic approaches to test the voracity of this so-called "+2"rule" for lipoprotein sorting and beginning to investigate the molecular explanation for exceptions to this rule that we and others have recently discovered.

Session II
Chair: Scott J. Hultgren

Structural and functional studies of the bacterial protein-translocation complex

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The Sec protein complex is localised in biological membranes to provide an essential and ubiquitous route for the translocation of secretory and membrane proteins. In bacteria, this transporter is formed by oligomers of a heterotrimeric membrane protein complex consisting of subunits SecY, SecE, and SecG. Translocating proteins are driven through the complex by an ATPase SecA or during their synthesis from bound ribosomes. The structure of the *E. coli* SecYEG assembly has been determined at 8 Å resolution (Breyton *et al.*, 2002). The three-dimensional map calculated from two-dimensional SecYEG crystals reveals dimers of SecYEG within a phospholipid bilayer. This may represent the closed state of the protein-conducting complex. This dimer has been found to associate with translocating polypeptide (Bessonneau *et al.*, 2002) and also with both monomeric and dimeric forms of the partner protein SecA. Complexes of the core complex together with peptides, SecA or with ribosomes are under current investigation.

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Protein and DNA traffic in bacterial conjugation

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Genetic determinants for bacterial conjugation contain a type IV protein secretion system (T4SS) that is essential for the conjugation process (Zechner *et al.*, 2000). There are different views about the role of T4SS: (i) they could transport DNA and proteins, (ii) they could transport proteins and nucleoproteins, and (iii) they transport only proteins. We favour the third alternative and have proposed a shoot and pump conjugation model to explain it. In our view, conjugation occurs in two steps (Llosa *et al.*, 2002). In step I, or ***shoot step***, the relaxase (which is called TrwC in our model plasmid R388) is shot to the recipient by the T4SS. In step II, or ***pump step***, the relaxase covalently bound DNA is pumped to the recipient by means of the DNA transporter TrwB. Therefore, detailed analysis of proteins TrwB and TrwC is key to understand the conjugation mechanism. We have undertaken a project to understand the structure-function relationship of these two proteins.

The three dimensional structure of TrwB (Gomis-Ruth *et al.*, 2001; Gomis-Ruth *et al.*, 2002) shows a hexameric molecule of identical subunits, which is structurally related to the F1subunit of ATP synthase. The structure immediately suggests that TrwB should be playing a motor role in a given conjugation step. Site-directed mutagenesis has underscored the importance of the ATP-binding site for TrwB function. Nevertheless, purification and analysis of the wild type protein has not resulted yet in an active ATPase (Hormaeche *et al.*, 2002). TrwB interacts with the relaxosome components TrwA and TrwC, and with the T4SS component TrwE. These interactions underline the coupling role that is additionally played by TrwB (Llosa *et al.*, 2003).

The three dimensional structure of the relaxase protein TrwC in complex with a single-stranded DNA piece of the *nic* site has been recently achieved (Guasch et al., submitted). TrwC binds supercoiled DNA at a specific site of the *oriT* called *nic*. Upon binding, it distorts the DNA so that it becomes single-stranded in the immediate vicinity of *nic*. TrwC-*nic* site complexes are extraordinarily stable, with half lives in excess of 10 hours. The structure nicely explains tight DNA binding, and allows us a first view at the DNA processing active site. Besides, site-directed mutagenesis of invariant residues in the TrwC protein family indicated a key role for six individual amino acids. The six of them are found in close proximity to the active site. Analysis of the structures of three different complexes, together with the results of site-directed mutagenesis, allowed us to propose a model for the mechanism of conjugative DNA processing. Our model not only explains conjugative DNA

processing, but provides also essential clues into the mechanism of initiation of rolling-circle replication (Grandoso *et al.*, 2000; Guasch et al., submitted).

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Two-partner secretion: the model of filamentous haemagglutinin of *Bordetella pertussis*

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The Two-Partner Secretion (TPS) pathway in Gram-negative bacteria appears to be dedicated to the secretion of large (> 100 kDa) proteins, mostly in pathogens. In the TPS systems, the proteins to be secreted, or TpsA proteins, are transported across the outer membrane via their specific TpsB partners [1]. The hallmarks of the TPS pathway are the N-proximal, highly conserved "TPS domains" of the secreted TpsA proteins and the outer membrane transporters of the TpsB family (TC # 1.B.20) [2]. The secretion of filamentous haemagglutinin (FHA) by *Bordetella pertussis* is a model for the TPS pathway. A major adhesin of *B. pertussis*, FHA is synthesized as a large, 367-kDa preproprotein called FhaB, which is translocated across the outer membrane by its specific transporter, FhaC and then undergoes an extensive C-terminal maturation at the cell surface.

FhaC likely forms a β-barrel channel in the outer membrane [3]. Following a specific interaction between FHA and FhaC at the periplasmic side of the outer membrane, FHA is translocated to the bacterial surface by a yet unknown mechanism. Because of the conservation of the TPS domain, we have hypothesized that it is involved in the molecular recognition between the two partner proteins. By X-ray crystallography, we have recently solved the tertiary structure of an N-terminal truncate of FHA comprising the TPS domain and a few of the numerous 19-residue repeats that make up the central part of FHA. This FHA derivative forms a β-helix, with the TPS domain possibly serving as a nucleator for the folding of the rest of the protein (our unpublished data). The determinants that interact with FhaC remain to be identified.

Like Sec-dependent secretory proteins, FhaB is synthesized with a cleavable, N-terminal signal peptide, which is however significantly longer (71 residues) than typical signal peptides. This extended signal sequence is similar to those of a subset of autotransporters and TpsA proteins, with a conserved "N-terminal extension" and a high number of positive charges in the N region [4]. The distinctive functions of such signal peptides have remained unclear. We have obtained indications that FHA is targeted in a co-translational manner in *B. pertussis* (our unpublished data). This contrasts with secretory proteins bearing classical N-terminal signal peptides, which use a post-translational, chaperone-dependent targeting pathway [5,6].

The mature protein corresponds to the N-terminal 230 kDa of FhaB. The protease involved in the maturation of FhaB, SphB1, is an autotransporter with a subtilisin-like N-terminal "passenger" domain [7]. SphB1 also catalyses its own proteolytic maturation between its passenger and membrane domains, although the mature protease remains associated to the bacterial surface. We have shown that SphB1 is a lipoprotein, and this modification mediates its surface retention after maturation [8]. The membrane localization of SphB1 is required for the processing of FhaB. Mature FHA partitions between the cell surface and the extracellular milieu, and its release may participate in the establishment of the infection [9].

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The filamentous type III secretion system of EPEC: protein interactions and structural analysis

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Many pathogenic bacteria exploit surface host cell proteins in order to bind to eukaryotic cells, a process which is essential for colonisation of a specific niche within the animal host. In contrast, a defined group of extracellular enteric pathogens [including enteropathogenic (EPEC) and enterohaemorrhagic *E. coli* (EHEC)], which colonise the gut while producing a characteristic attaching and effacing (A/E) lesion (Frankel *et al.*, 1998), does not rely on existing host cell receptors to establish intimate bacterium - enterocyte contact, but instead delivers its own receptor (Tir) to the enterocyte plasma membrane (Kenny *et al.*, 1997). The apparatus assembled by the bacterium to deliver Tir to the host cell is a filamentous type III secretion system (TTSS) (Knutton *et al.*, 1998; Daniell *et al.*, 2001).

The type-III secretion apparatus is a multi-component organelle assembled from the products of approximately 20 genes. Many components are broadly conserved amongst both virulence and flagellar TTSSs (Hueck, 1998). The secretion apparatus that can be isolated from bacterial membranes and visualised by transmission electron microscopy is referred to as the needle complex (NC). Assembly of the NC occurs in stages; initially, in a *sec*-dependent manner, the membrane-bound components are exported to form the foundation of the NC (consisting of stacked membrane rings joined by a central rod). This is followed by assembly of the inner membrane-associated machinery, which enables secretion of components constituting the 'needle' and the more distal components of the apparatus, including translocator proteins; translocator proteins interact directly with the eukaryotic plasma membrane to facilitate translocation of effector proteins across three membranes in a single step.

In EPEC one protein, EscC (a member of the secretin superfamily), is known to be located in the outer bacterial membrane, where it believed to form a large homomultimeric ring complex. Based on homology with other systems, part of the EscC ring is thought to project into the periplasm where it can interact with inner membrane proteins to form the central rod observed by electron microscopy. Other conserved TTSS proteins, including EscR, S, T, U and V, are predicted to span the inner membrane, although their precise function and localisation within the apparatus is currently unclear. The EPEC TTSS also has several cytoplasmic components including EscN, a highly conserved ATPase, the LEE-encoded regulator (Ler) and several chaperones. We have recently performed a global yeast two-hybrid system screen to identify pair-wise interactions between components of the EPEC TTSS (Creasey *et al.*, 2003) and these results will be presented.

The needle component of the EPEC TTSS is composed of EscF (Wilson *et al.*, 2001), a protein which shares a high level of similarity to needle proteins from other TTSSs. However, unique to the EPEC TTSS is the fact that it consists of a filamentous structure (EspA filament) which extends from the distal end of the EscF needle (Sekiya *et al.*, 2001; Daniell *et al.*, 2001) and forms a direct link between the bacterium and the host cell (Knutton *et al.*, 1998). Following EspA filament-mediated cell adhesion and protein translocation, expression of *espA* and other TTSS genes is markedly down regulated.

The three-dimensional structure of the EspA filament shows that it consists of a helical tube of outer diameter *c.*120 Å containing a hollow central channel *c.* 25 Å in diameter with a continuous wall, which is likely to contain the proteins within the channel during transfer (Daniell *et al.*, 2003). If, as we hypothesised, the terminal regions of EspA form the inner core of the filament, the uncharged, hydrophilic residues in these regions may be exposed to the channel providing an environment less likely to impede transport. Evidence suggests that EspA is the major component of this filamentous structure, although the translocator protein EspD, which is presumed to be exported through the filament, is also required for filament assembly (Knutton *et al.*, 1998). EspD in complex with a second translocator, EspB, is predicted to form a pore in the host cell membrane that facilitates subsequent entry of virulence proteins. We have recently advanced our functional characterisation of these translocator proteins and the results of these studies will be shown.

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The Tat protein transporter of *Escherichia coli*

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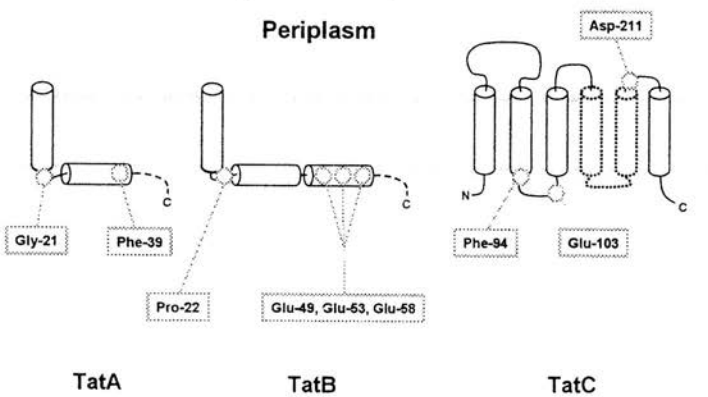
The bacterial Tat (**t**win **a**rginine **t**ranslocation) protein transport pathway has the function of exporting folded proteins across the cytoplasmic membrane. This is a particularly challenging task since it requires the provision of a transmembrane channel that is large enough to allow the passage of structured macromolecular substrates of up to 60Å in diameter but that is able to maintain the impermeability of the membrane to ions (including protons). In addition the Tat transporter transduces the energy of the transmembrane proton electrochemical gradient to effect unidirectional movement of the substrate through the channel. We are pursuing structure-function analysis of the Tat transporter with the aim of understanding its unusual molecular mechanism.

In *Escherichia coli* the *tatABC* operon codes for three integral membrane proteins that are the minimal essential components of the Tat system. TatC is a polytopic membrane protein while TatA and TatB are sequence-related, though functionally distinct, monotopic membrane proteins with a membrane-extrinsic cytoplasmic domain (see Figure). In the resting state the three Tat proteins form two distinct, high molecular mass complexes, one containing predominantly TatA and the other predominantly TatBC. The TatBC complex contains a Tat signal peptide binding site and thus acts as a receptor complex while TatA is thought to form the translocation channel. Evidence from a Tat-analogous system of plant thylakoids indicates a dynamic interaction between these two complexes during substrate translocation.

Our recent data on the Tat proteins will be presented.



Predicted secondary structure and topological organisation of essential Tat components in *E. coli*. The presence of the two predicted transmembrane helices in TatC that are depicted using a dotted line is not supported by some recent experimental data from the group of Long-Fei Wu (*FEBS Lett.* **525:** 65-70). The positions of some inactivating point mutations are shown.

A Recent Review:

Berks, B.C., Palmer, T., and Sargent, F. (2003) The Tat protein translocation pathway and its role in microbial physiology. *Adv.Microbiol.Physiol.* **vol. 47**.

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Hicks, M.G., de Leeuw, E., Porcelli, I., Buchanan, G., Berks, B.C., and Palmer, T. (2003) The *Escherichia coli* twin-arginine translocase: conserved residues of TatA and TatB family components involved in protein transport. *FEBS Lett.* **539:** 61-67.

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Session III
Chair: Tracy Palmer

Bacterial fibers and their role in disease

Scott J. Hultgren

We have elucidated the structure, function and mechanism of action of bacterial factors involved in host pathogen interactions, host responses and bacterial subversion mechanisms that facilitate persistence using a blend of a genetic system with X-ray crystallography, animal models, cell biology, biochemistry, high-resolution electron and video microscopy, functional genomics and immunology. These approaches allowed us to discover the molecular basis of a molecular machine found in hundreds of pathogenic microbes called the chaperone/usher pathway that facilitates the assembly of adhesive fibers, called pili that participate in host-pathogen interactions. We also have discovered that *E. coli* produce amyloid-like fibers called curli. Fundamental questions addressing the structural basis of the protein-protein interactions involved in amyloid formation were studied. This work will provide clues into the structure and function of curli and have implications for the pathology of Alzheimer's and other amyloid diseases.

We also used a multidisciplinary approach to investigate the function of these fibers and other virulence factors in *E. coli* pathogenesis. Our studies have changed the clinical paradigm of urinary tract infections (UTIs) and are leading to new and better treatments and clinical diagnoses. We solved the structural basis of how pilus adhesins of *E. coli* recognize receptors on uroepithelial cells and discovered an elaborate network of molecular cross-talk that occurs as a consequence of these interactions. Our work revealed that *E. coli* virulence depends on invasion into the superficial umbrella cells of the bladder where the bacteria rapidly replicate and form intracellular biofilms that we termed pods. The intracellular biofilm protects the bacteria from host responses and antibiotics thus allowing the bacteria to persist. We have sequenced the UPEC genome and are currently using a multitude of approaches to try to understand the molecular basis of chronicity and recurrence. This work is spawning new technologies to design novel vaccines and anti-microbial therapeutics that will block the ability of bacteria to adhere to host tissues and thus prevent their ability to establish infections.

To translocate or to insert? How preprotein translocase and YidC co-operate in the insertion of proteins into the cytoplasmic membrane

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Preprotein translocase is an enzyme complex that mediates the translocation of unfolded secretory proteins across the cytoplasmic membrane. The core of the translocase consists of two domain, i.e., a motor domain which constitutes the SecA ATPase, and a transmembrane channel formed by an oligomeric assembly of the SecY, SecE and SecG polypeptides. This core domain of translocase interacts with another heterotrimeric integral membrane protein complex, i.e, the SecDFyajC complex, but the exact function of this accessory complex is not known. Translocase is also involved in the co-translational insertion of most inner membrane proteins. During their membrane insertion, these nascent chains transiently interact with the YidC protein, a bacterial homolog of the mitochondrial OXA1p that is essential for the cell viability. YidC itself also transiently interacts with the core domain of the translocase, but most of the YidC is translocase unassociated while a fraction forms a stable complex with the SecDFyajC proteins. Remarkably, whereas YidC appears to be dispensable for the assembly of Sec-dependent membrane proteins, it is essential for the proper integration of some phage coat proteins. These latter proteins insert into the membrane independently of the translocase.

In an effort to understand the essential function of YidC and its interplay with the translocase in more detail we have studied the physiological consequences of YidC depletion. The loss of YidC rapidly and specifically induced the Psp stress response, which is accompanied with by a reduction of the proton motive force. The latter is due to defects in the functional assembly of cytochrome *o* oxidase and the F_1F_0 ATPase complex, which is reminiscent of the effects of mutations in the *yidC* homologue *OXA1* in the yeast mitochondrial inner membrane. The integration of CyoA (subunit II of the cytochrome *o* oxidase) and F_0c (membrane subunit of the F_1F_0 ATPase) appeared exceptionally sensitive to depletion of YidC, suggesting that these IMPs are natural substrates of a membrane integration and assembly pathway in which YidC plays an exclusive or at least pivotal role.

In a further effort to understand the mechanism of membrane insertion, we have reconstituted this process for various membrane proteins using proteoliposomes containing the purified SecYEG complex and YidC protein. FtsQ is a monotopic integral membrane protein with an N-terminal transmembrane domain and a C-terminal periplasmic domain. Ribosome-bound nascent chains of FtsQ are targeted to the SecYEG complex via the SRP pathway. The transmembrane domain of nascent FtsQ inserts into membrane via the SecYEG

complex but independently from SecA. During insertion, it transiently interacts with YidC. Membrane insertion of full-length FtsQ requires in addition to SecYEG, SecA and a transmembrane electrical potential. The latter two are essential for translocation of the large periplasmic domain of FtsQ. The presence of co-reconstituted YidC has an inhibitory effect which suggests that YidC delays the membrane insertion of the Sec-dependent membrane protein. We have also reconstituted the co-translocational membrane insertion of the F_0c subunit of the F_1F_0 ATPase complex. The results of these studies will be discussed. Taken together, our data demonstrate that membrane protein insertion can be reconstituted with only a minimal set of purified Sec-components.

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The complexity of bacterial autotransporters

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Autotransporters (ATs) are a family of secreted proteins that contain all the necessary elements for translocation across the outer membrane (OM) within their own polypeptide sequences. Members of the AT family contain a common modular structure with a C-terminal transporter domain that inserts into the OM and that translocates the linked N-*passenger* module (i.e. protease, adhesin, cytotoxin etc.) into the extracellular medium. We have investigated the molecular mechanism of AT secretion using as a model the IgA1 protease from *Neisseria gonorrhoeae*. A 6xHis-derivative of the 45 kDa C-terminal domain of IgAP (C-IgAP) was expressed in *E. coli* cells and purified in its native, functional state from the OM. Biochemical and electron microscopy studies employing the purified protein, as well as *in vivo* crosslink experiments with *E. coli*-expressing cells, showed that this autotransporter C-domain forms a ring-like oligomeric complex of at least 300 kDa containing a central cavity of ~2 nm in diameter through which N-domains appear to be translocated (Fig. 1).

In a different set of experiments the natural N-passenger of IgAP was substituted by various types of immunoglobulin (Ig) domains (~2 nm size). The Ig N-domains of these hybrid proteins were efficiently translocated toward the cell surface in a folded and active state. Periplasmic protein chaperones, such as DsbA and FkpA, fold the Ig domains prior to their translocation across the OM. Furthermore, we found that large N-passengers containing a string of three folded Ig domains were secreted by C-IgAP with no loss of efficacy. On the contrary, a single domain of GFP (~4 nm) could not be exported by C-IgAP in a folded form once exported into the periplasm by the TAT pathway. These data suggest that the maximum size for a protein domain that can be translocated by C-IgAP in a folded state is in the range of 2 to 3 nm.

Taken together our data indicates that the secretion mechanism of AT may have important similarities with other bacterial export systems that rely on multimeric OM complexes (e.g. the secretins and fimbrial ushers) and the action of periplasmic chaperones.

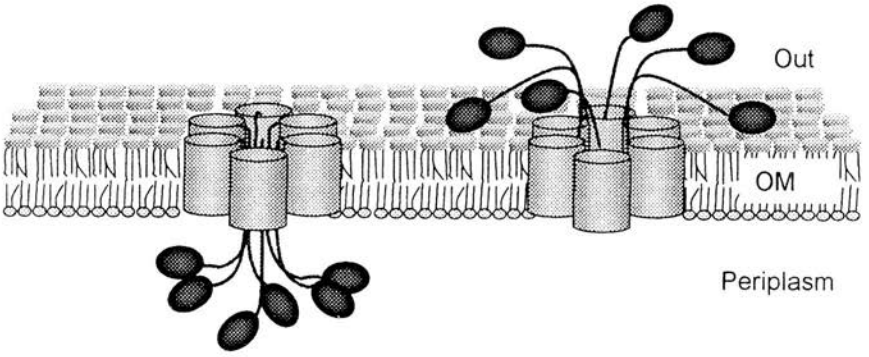

Figure 1. Proposed model for the secretion of autotransporters. The C-domain assembles in the OM an oligomeric complex with a central hydrophilic pore of ca.2 nm through which N passenger domains are translocated (Veiga et al. 2002). Folded N- domains of 2 nm can be efficiently transported by this complex.

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Programming secretion systems for releasing active proteins to the environment

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Engineering *in situ* release of active proteins to the niches where bacteria naturally inhabit is an endeavour of considerable biotechnological interest. We have exploited the export signals of the type I and of type V secretion systems for transtopic production of various types of active polypeptides destined to have an effect in the surrounding environment. A few cases will be presented: [i] A simple method for the non-toxic, specific and efficient secretion of active single-chain Fv antibodies (scFvs) into the supernatants of *E. coli* cultures was based on hemolysin transport system (Hly) of *E. coli* that specifically secretes the target protein from the bacterial cytoplasm into the extracellular medium without a periplasmic intermediate. The culture media that accumulate these Hly-secreted scFvs can be used in a variety of immunoassays without purification. When Hly fusions were made to anti-viral scFvs, these culture supernatants basically behaved as immune sera, able to counteract the effect of viral particles in infection assays. Since type I secretion systems appear to tolerate somewhat bulky proteins, work is in progress to increase the neutralizing activity of such secreted scFvs by engineering them as dimers with heterologous dimerization domains. [ii] Similarly, fusions of single-chain antibodies (scFvs) to the autotransporter ß domain of the IgA protease of *Neisseria gonorrhoeae* were instrumental to deliver virus-neutralizing activity to the medium. *Escherichia coli* cells displaying scFvs against the transmissible gastroenteritis coronavirus (TGEV) on their surface blocked *in vivo* the access of the infectious agent to cultured epithelial cells. These result raise prospects for anti-viral strategies aimed at hindering the entry to target cells with bacteria that naturally colonize the same intestinal niches. [iii] Hybrid proteins containing the AT domain of the IgA protease (IgAß) and the partner leucine-zippers of the eukaryotic transcriptional factors Fos and Jun were expressed in *Escherichia coli*. Such fusion proteins targeted the leucine-zipper modules to the cell surface. Cells displaying the Junß sequence flocculated short after induction of the hybrid protein. *E. coli* cells expressing separately Fosß and Junß chimeras formed consortia whose strain composition reflected the degree of expression of each partner of the corresponding leucine zippers. These associations were physically held by tight inter-cell ties caused by the protein-protein interactions of matching dimerization domains and open the possibility of designing bacterial consortia *a la carte* for specific purposes (for instance, as microbial catalysts). [iv] The mouse metallothionein I (MT) has been targeted to the cell surface of the heavy-metal tolerant *Ralstonia eutropha* CH34 strain that is adapted to thrive in soils highly polluted with metal ions. To this end, the DNA sequence encoding MT was fused to the AT of the IgA protease, which targeted the hybrid protein towards the bacterial outer membrane. The resulting bacterial strain, named *R. eutropha* MTB, was found to have an enhanced ability for immobilizing Cd^{2+} ions from the external media. Innoculation of Cd^{2+}-polluted soil with *R. eutropha* MTB decreased significantly the toxic effects of the heavy metal on the growth of tobacco plants.

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Adapting Type I secretion of the crystalline surface layer protein of *Caulobacter crescentus* for protein secretion and display

John Smit

C. crescentus is a harmless freshwater bacterium that produces a very useful two-dimensional crystalline assembly on its outer surface, composed of a single highly expressed protein. Because it uses the flexible Type I secretion mechanism for export of this protein, we have been able to adapt the secretion system to enable secretion of a wide variety of proteins (using only the secretion signal) or for display of peptides and proteins on the cell surface, using the entire S-layer gene. Display of peptides of 50-150 amino acids is commonly accomplshed and in some cases we can present insertions of more 650 amino acids. Because of the crystalline nature of the S-layer successful presentation occurs at high density--about 40,000 copies/cell. We have also developed small, high copy number plasmid vectors to rapidly produce peptide, gene fragment or antibody display libraries. The high copy number and the fact that it is a bacterial display system allows ready adaptation to flow cytometry as a rapid means to detect clones of interest. Protein secretion by fusion to the C-terminal secretion signal also has a built-in purification mechanism due to the unusual aggregate forming properties of the S-layer monomer secretion signal. Other applications of the S-layer display in development include whole cell vaccines for veterinary or anti-cancer applications, 'particles' with catalytic or serodiagnostic capabilities, and pathogen/toxin sorbants or anti-infectives.

Session IV
Chair: Guy R. Cornelis

Chaperone, folding and type I secretion

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Type I secretion is a simple mechanism for a protein to be secreted by a Gram-negative bacterium. It involves a secretion apparatus made up of three distinct functions localized in the cell envelope: two proteins are localized in the cytoplasmic membrane, one of them being of the ABC class, whereas the other is from the membrane fusion class; the last one is an outer membrane protein of the TolC class.

Many proteins of different classes are secreted by this system and a given bacterium can secrete several distinct proteins by this system, with either specific systems for each different class of proteins or with components common to several systems.

The HasA hemophore of *Serratia marcescens* is a 188 aminoacids long protein secreted under iron starvation conditions by an ABC pathway. Its function for the bacterium is to catch heme thanks to its very high heme affinity (10^{-11}M) and to return it to a specific outer membrane receptor that allows heme internalization and its subsequent use as an iron source.

As most proteins secreted by this pathway, HasA possesses a C-terminal secretion signal, necessary and sufficient to trigger the ordered association of the secretion complex with the successive recruitment of the ABC protein and the MFP and the OMP.

We have shown that in the absence of the secretion function HasA is able to fold inside the cytoplasm as evidenced by the presence of a characteristic absorption band at 407nm due to heme binding. This folded form is no longer secretion competent. Furthermore this folded form is still able to interaqct with the ABC tranporter as it inhibits the secretion of newly synthesized HasA. This inhibition does not involve the C-terminal secretion signal since a HasA variant devoid of the C-terminal secretion signal and still able to fold into the cytoplasm inhibits the secretion of newly synthesized HasA. This indicates the existence of another site on the ABC protein besides the site for the C-terminal secretion signal. A collection of HasA mutants is used to determine this site of interaction.

We also showed that SecB, the cytoplasmic chaperone of the Sec system is involved in HasA secretion, both in the reconstituted system in *E. coli* and in the original host *S.*

marcescens. SecB is thus involved into two distinct processes, Sec system and ABC protein secretion. We also showed that *in vitro* folding of denatured HasA is extremely fast and that SecB slows down HasA folding to a very large extent. We have constructed *in vitro* HasA mutants affecting the folding rate of HasA. These mutants are secreted largely independently of SecB although their overall fold and their functionality is not affected. We are presently looking into more details to this interaction of HasA with SecB.

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Type III secretion system of the plant pathogen *Pseudomonas syringae*

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The Hrp type III secretion system of *Pseudomonas syringae* injects effector proteins through plant cell walls and into the cytosol and is essential for plant pathogenicity. A combination of experimental and computational approaches have been taken to identify all of the TTSS effector genes in the complete genome sequence of *P. syringae* pv. *tomato* DC3000, a pathogen of tomato and the model plant Arabidopsis (1, 2). Over 40 proteins have now been confirmed to travel the TTSS pathway in DC3000 based on immunoblot analysis of culture supernatants and on bioassays involving avirulence protein (AvrRpt2 lacking native targeting signals) or adenylate cyclase (Cya) translocation reporters. Additional effector gene candidates have been identified in the genomic sequence of DC3000 based on amino acid patterns in the N-terminal 50 residues (4). We have used site-directed mutagenesis of known effectors and translocation tests of candidate effectors and relevant non-candidates to test the importance of these predictive patterns in targeting proteins to the TTSS. In general, effectors are weakly secreted by the TTSS in culture. In contrast, the HrpA pilus subunit and harpins are secreted more abundantly. The Hrp pilus is an essential conduit for effector translocation (3). The role of harpins is less clear, and analysis is complicated by apparent redundancy. Harpins are glycine-rich, cysteine lacking proteins that are secreted by the TTSS and can elicit the hypersensitive response (defensive programmed cell death) when exogenously applied to plant cells. Analysis of the DC3000 genome for genes that are in the Hrp TTSS regulon and that predict proteins lacking cysteine yielded the known harpins HrpZ and HrpW and two novel harpins, HopPtoP and HopPmaH$_{Pto}$, which were subsequently demonstrated to be TTSS-secreted and to elicit the hypersensitive response in tobacco leaves. Mutations in individual harpin genes have little effect on the translocation of effectors, possibly because of redundancy. Consequently, harpin function was explored by using the cloned cluster of *P. syringae* pv. *syringae* 61 TTSS genes carried on pHIR11, which encodes a single harpin, HrpZ, and enables nonpathogens, such as *P. fluorescens*, to translocate *P. syringae* effectors into plant cells. Deletion of *hrpZ* reduced the ability of pHIR11 to direct the translocation of AvrPto-Cya into plant cells in a conditional manner. We are also using nonpolar mutations in the *P. syringae* pv. *syringae* 61 TTSS gene cluster to identify additional components that contribute specifically to the translocation of effectors into plant cells. Regarding the activity of effectors in plant cells, we are focusing on HopPtoN, which we have shown to be translocated in a ShcN chaperone-dependent manner, to be a cysteine-protease, and to suppress programmed cell death in both plants and yeast.

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Novel aspects of protein secretion and adhesion in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a gram-negative bacterium, opportunistic pathogen, which is responsible for severe nosocomial infections that become chronic and lethal for patients with cystic fibrosis. The pathogenicity of the organism is associated with numerous virulence factors that include molecular machineries responsible for the release of toxins and enzymes (1) as well as for the assembly of adhesive structures such as pili or flagella (2).

The genome of *P. aeruginosa* has been completely deciphered in the early 2000 (3), and the informations provided have given access to extensive study in functional genomics. Firstly, the genome sequence has allowed the quick identification of mutated genes that gave a phenotype of interest. Secondly, the "in silico" identification of genes possibly involved in molecular process of interest has allowed the quick design of the corresponding mutants that could be phenotypically analyzed. We did perform such analysis by looking at two key phenotypes in bacterial infection that are adhesion and protein secretion.

We could reveal that *P. aeruginosa* possesses adhesive structures assembled by the chaperone-usher pathway, structures that were never described before in this organism. This system was called CupA (4). Interestingly two additional *cup* gene clusters could be found in the genome, and called *cupB* and *cupC*. In all three cases one single usher outer membrane component is associated with one or more fimbrial subunits and as many chaperones. The usher component allows presentation of the fimbrial subunit to the extracellular milieu and assembly into a pilus rod. Among the fimbrial subunits, it could be clearly distinguished between the putative rod-constituting component and the putative adhesin. Indeed, adhesins assembled *via* these Cup systems have the conserved fimbrial domain at their C-terminal end, whereas they present a unique domain at their N-termini which contains a specific binding pocket (5). We have shown that some of the Cup systems allow efficient binding on plastic whereas others have higher affinity for epithelial cells. In addition to the Cup systems many other systems might contribute to bacterial attachment and biofilm formation. One such system has been named Tad, for tight adherence, by reference to the original system identified in *Actinobacillus actinomycetemcomitans* (6). This system is very similar to the machinery that allows assembly of type IV pili (7), and is involved in the assembly of Flp pili that belongs to the so-called type IV-b class of pili. In both cases the assembly mechanism is drastically different as compared to the chaperone-usher pathway. It involves components belonging to highly conserved families of proteins implicated in various macromolecular transport systems. These include the so-called traffic ATPases and the outer membrane components called secretins.

Traffic ATPases and secretins are also involved in type II protein secretion in gram-negative bacteria. Type II secretion is a two-steps process in which initial translocation across the inner membrane is achieved *via* either the Sec or Tat machineries (8). The secreted proteins are then loaded onto the type II secretion machinery, or secreton, from the periplasmic side of the cytoplasmic membrane and finally released into the extracellular milieu. In *P. aeruginosa* the type II secreton is called Xcp and is involved in the release of numerous and distinct toxins and enzymes (1). The *P. aeruginosa* genome analysis revealed the existence of a gene cluster that did contain eleven genes, similar to the previously characterized *xcp* genes, that we named *hxc* for homologous to *xcp* (9). Whereas the Xcp secreton, is involved in the secretion of elastase, exotoxin A or phospholipases, the Hxc system directs specifically the secretion of two alkaline phosphatases. Intriguingly, it has been shown that the type II secreton could assemble pili-like structures that were called pseudopili. In *P. aeruginosa* such structures are detected upon overproduction of one of the secreton component, XcpT, which belongs to the GspG family (10). Such assembly mechanism is broadly conserved in gram-negative bacteria since it was originally observed with the Pul system in *K. oxytoca*, which is involved in pullulanase secretion (11). The mechanism and function of the pseudopilus assembly in the secretion process or in bacterial attachment and biofilm formation is a key question to be addressed. It is now obvious that several systems, including type II secretion and type IV piliation, involved very similar mechanisms that have evolved towards different function (attachment, motility, secretion).

The redundancy of the systems we just described helps the ability of *P. aeruginosa* to proliferate in numerous and different environments and hosts and we will further study how they cooperate or alternate during the various stages of colonisation.

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Type III protein secretion in *Pseudomonas syringae*

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Pseudomonas syringae causes numerous diseases in plants. This pathogen uses the *hrp* gene-encoded type III secretion system to inject more than 30 effector proteins into plant cells to modulate host susceptibility. In this talk, several aspects of the *P. syringae* type III secretion system will be reviewed, including the evidence for the type III secretion function of *hrp* genes, the intracellular action of effector proteins, the function of the Hrp pilus in type III secretion, the identification of a chaperone protein, and the virulence activities of type III effector proteins on Arabidopsis signaling and metabolism.

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The physiological role of the *Escherichia coli* twin-arginine translocase

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A group of bacterial extracytoplasmic and inner membrane proteins are synthesised as precursors with N-terminal twin-arginine signal peptides bearing a conserved SRRxFLK amino acid sequence motif. Substrate proteins bearing twin-arginine signal peptides are transported in *fully folded* conformations by the membrane-embedded twin-arginine protein transport (Tat) system using energy provided by the transmembrane Δp. In *Escherichia coli* approximately twenty-six known or putative passengers on the Tat pathway have been identified. This is a modest number considering approaching 20% of *E. coli* proteins may be exported from the cell cytoplasm by other routes, however the Tat 'secretome' contains key players in a number of biochemical processes. Here we review our recent work aimed at understanding the physiological role of the *E. coli* twin-arginine translocase by studying the assembly and trafficking of endogenous Tat substrates.

In *E. coli* the *tatABC* operon codes for three integral membrane proteins that are the minimal essential components of the Tat system. Genetic inactivation of the *tat* operon results in defects in cell division, outer membrane assembly, biofilm formation and respiration of various compounds including molecular hydrogen. We have shown that the defects in cell division, biofilm formation and outer membrane assembly result from the mis-localisation of two Tat-dependent cell wall amidases (AmiA and AmiC) in the *tat* mutant. The inability of the *tat* mutant to respire hydrogen gas is as result of mis-localisation of two Tat-dependent [NiFe] hydrogenases (HyaAB and HybOC). The *E. coli* [NiFe] hydrogenase-2 isoenzyme consists of a heterodimer of an Fe-S cluster-binding subunit (HybO) together with a subunit that binds the Ni-Fe active site cofactor (HybC). The HybOC dimer is targeted to the Tat translocon as a pre-formed unit by a Tat signal peptide located on only one subunit (HybO). Substitution of the HybO Tat signal peptide with that of the molybdoenzyme TorA resulted in unco-ordinated assembly and targeting processes and thus reduced [NiFe] hydrogenase-2 activity. Interestingly, the mutant phenotype could be significantly rescued by overexpression of the *torD* gene encoding the TorA system-specific chaperone. This experiment points directly to a role for TorD in Tat signal peptide binding and highlights the existence of chaperone-mediated 'proofreading' processes at work during the assembly of complex Tat substrates. In addition we have shown that [NiFe] hydrogenase-2 is the archetype of a new class of Tat-dependent (Sec-independent) integral membrane proteins. Our most recent data on the mechanism of membrane protein integration by the Tat translocase will be presented.

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Components and dynamics of Type IV pilus expression linked to organelle structure and function

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Type IV pili (Tfp) represent a unique class of proteinaceous appendages defined by shared structural features, associated phenotypes and a conserved biogenesis pathway which are found in extremely diverse groups of Gram negative species. Their biogenesis involves a large number of components, the genes for which are most often distributed throughout the genome. Tfp are associated with many diverse phenotypes including adherence to both mammalian cells and inanimate surfaces, phage sensitivity, twitching motility and competence for natural genetic transformation. Elucidation of the biogenesis pathway and direct biophysical studies have revealed that Tfp are dynamic structures which grow and retract and that significant physical force is generated during these processes. Tfp can thus be considered to be a polymerization motor that generates force in a manner analogous to that associated with actin polymerization. Neisserial Tfp retraction has been implicated in motility, the uptake of DNA during natural genetic transformation and the initiation of a unique signal transduction system in human host cells.

The goal of this talk is to provide an update of the gonococcal Tfp system and describe the results of new studies. The latter will include studies of proteins structurally related to the pilin subunit, their influence on the dynamics of Tfp expression, and their roles in pilus-associated function. In addition, studies exploring the relevance of the neisserial findings to other Tfp expressing organisms will be presented.

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Session V
Chair: Vassilis Koronakis

The Yersinia "Ysc-Yop" weapon, a supramolecular structure allowing proteins to cross three membranes in a row

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Several animal and plant pathogenic Gram negative bacteria use a "type III secretion" system to attack their host. These systems are activated by contact with a eukaryotic cell membrane and they allow bacteria to inject bacterial proteins straight across the two bacterial membranes and the eukaryotic cell membrane, and subvert the target cell(*1*, *2*). The Yop virulon, an archetype of these systems, allows bacteria from the genus *Yersinia* to resist the primary immune response of the infected host by neutralizing phagocytes. It consists of a secretion apparatus, called injectisome, made of 25 Ysc proteins and a set of 12 proteins called Yops that are exported by the Ysc apparatus.

The injectisome, derived from the flagellum, consists of an intrabacterial body, two transmembrane rings and a 60-nm needle protruding outside the bacterium(*3*). The length of the needle is controlled by protein YscP, a protein which is itself secreted by the apparatus(*4*). Translocation of the effectors across the cell membrane cannot be achieved by the injectisome itself, in spite of the fact that it terminates with a needle. Translocation requires other secreted proteins, namely YopB(*5*) and YopD(*6*) which form pores(*7*, *8*) in the target cells. Pore formation also requires LcrV(*9*). To demonstrate this, one has to circumvent the fact that LcrV also exerts a regulatory role on the synthesis of YopB and YopD.

According to their permeability, the translocation pores have an approximate diameter of 2 nm but they have not been physically characterized yet. These pores are only detectable with *Y. enterocolitica* mutant strains devoid of all the effectors, which suggests that the translocating effectors obstruct the translocation channel. However, pore formation can also be detected with simple *yopN* mutants. These mutants secrete the effectors but the effectors are not translocated(*6*, *10*). This suggests that YopN might form a link between the injectisome and the pore.

In conclusion, successful delivery of the effectors involves the injectisome ending up with a needle, YopB, YopD and LcrV forming a pore in the target cell and YopN acting as a hypothetical connector between the two structures.

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Structure and function of the neisserial autotransporter NalP

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Gram-negative bacteria have developed many different pathways to secrete proteins across the double membrane into the extracellular medium. Perhaps the simplest of these pathways is the autotransporter system (1). Autotranporters are synthesized with a signal sequence for transport across the inner membrane via the Sec system. The subsequent transport of the N-terminal passenger domain across the outer membrane is mediated by a C-terminal extension, the translocator domain. The secreted passengers may have various functions at the cell surface or in the extracellular medium, often contributing to the virulence of pathogenic bacteria (2).

The first autotransporter described and studied into detail was IgA protease from *Neisseria gonorrhoeae* (3). When the genome sequences of two *Neisseria meningitidis* strains became available, we decided to search them for new autotransporters genes, hoping to discover novel vaccine candidates. In this study (4), we found eight different autotransporter genes, including the gene for IgA protease. Most of the proteins encoded by these genes showed homology to known virulence factors, such as the adhesins Aida-I from *Escherichia coli* and Hap and Hia from *Haemophilus influenzae*. We decided to focus our studies on one of these autotransporters, which we designated NalP.

NalP (for **n**eisserial **a**utotransporter **l**ipo**p**rotein) contains a lipobox (LSA↓C) at the end of the signal sequence, suggesting that it is lipidated. Indeed, lipidation could be demonstrated, but the secreted 70-kDa domain of NalP lacks the lipid moiety, due to additional N-terminal processing. A *nalP* mutant showed a drastically altered profile of secreted proteins. The most abundant secreted protein of *N. meningitidis* is IgA protease, which appears in the extracellular medium of the wild-type strain in two different forms, a high molecular weight form representing the entire passenger domain including the C-terminal α-peptide, which contains nuclear localization signals, and a smaller form, which lacks the α-peptide. In the *nalP* mutant, only the lower molecular weight form was detected (5). Apparently, expression of NalP mediates the release of IgA protease from the cell surface with the α-peptide attached. Similarly, App, a homologue of the adhesin Hap of *H. influenzae* was found to be released with an α-peptide attached only when NalP was expressed. The serine-protease active site of NalP was required for modulating the processing of these autotransporters. We propose that NalP can process App and IgA protease and hypothesize that this function of NalP could contribute to the virulence of the organism.

Two models for the transport of the passenger domain of autotransporters across the outer membrane have been proposed. Initially, it was proposed that the translocator domain

inserts as a β-barrel into the outer membrane, thereby forming a pore through which the passenger domain is transporter (6). More recently, it was reported that the translocator domain of IgA protease, expressed in *E. coli*, forms multimeric ring-shaped structures with a central cavity, and it was suggested that the passenger domains are transported through this central channel (7). To gain insight in the structure and function of the passenger domain of NalP, we have expressed it as inclusion bodies in *E. coli* and refolded it in vitro (8). The refolded protein showed pore activity in planar lipid bilayers, consistent with its channel function. The crystal structure of the protein was resolved (8). The structure showed a 12-stranded antiparallel β-barrel, exposing hydrophobic residues to the lipid environment of the membrane and hydrophilic residues to the interior, thereby creating a hydrophilic channel. This channel was largely occupied by the N-terminal segment of the crystallized protein, which forms a long α-helix, running from the periplasmic side to the extracellular side of the membrane, where, in the intact autotransporter, the passenger domain would be attached. This structure is consistent with the transport of the passenger through the β-barrel formed by the translocator domain, and inconsistent with the model in which the passengers are transported through a central channel in a multimer of translocator domains. Alternatively, since the pore in the β-barrel is rather narrow and does not allow for the transport of any folded domain, we propose that the passengers could be transported through an entirely different channel, i.e. a channel formed by Omp85 of the outer-membrane-protein-assembly machinery, on which the autotransporter pathway is dependent (9). In this model, the translocator domain may just function as the recognition signal for the Omp85 system. If this model is correct, the name "autotransporter" for the pathway would be inappropriate.

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An ATP-binding cassette (ABC) complex promotes translocation of the dispersin coat protein in enteroaggregative *Escherichia coli*

Junichiro Nishi, Jalaluddin Sheikh, Frederick R. Blattner, and James P. Nataro*

Enteroaggregative *Escherichia coli* (EAEC) is an emerging enteric pathogen defined by a characteristic aggregative adherence (AA) to HEp-2 cells in culture. An empirically derived DNA probe (the "AA probe") has been used to detect EAEC strains; however, potential gene products encoded by the probe have been heretofore unidentified. Here, we have characterized the region corresponding to the AA probe locus in EAEC virulence plasmid pAA2.

The locus comprises a cluster of five genes (designated aatPABCD), two of which exhibit significant identity to genes involved in ABC-dependent transport. Among the proteins predicted to be encoded by the Aat cluster are a permease homolog (AatP), and ATP-binding protein homolog (AatC) and a novel protein (AatA) which localizes to the outer membrane independently of the ATP-binding protein. The function of the Aat cluster cannot be predicted based on in silico analyses. We have recently characterized a novel protein capsule of EAEC strains, which promotes dispersal of EAEC on the intestinal mucosa. We show here that translocation of the subunit of the protein capsule, called dispersin (or Aap), is facilitated by the Aat ABC transporter locus. We also show that, like the dispersin capsule, transcription of the aat cluster is dependent on AggR, a regulator of virulence genes in EAEC. The aat cluster is a novel ABC transporter which may play a role in the pathogenesis of EAEC by promoting dispersin translocation.

Transport, export, and assembly of flagellar proteins

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Bacterial flagellum consists of more than 20 component proteins, forming major three substructures: the basal body, hook, and filament. The order of assembly of each component is well understood. The flagellum grows from bottom to tip, piling up one substructure onto another. However, in a substructure (eg. the filament), the subunit (flagellin) assembles distally, adding a new subunit to the tip of a growing substructure (filament) by the help of a capping protein (HAP2). Since most of the flagellum is exposed outside, the component proteins are secreted and assemble outside of the cell.

The flagellar secretion belongs to the so-called type III secretion system (TTSS), which is characterized by the fact that secreted proteins have no signal sequences. The flagellar system is known to consist of an export apparatus and soluble components (chaperones and an ATPase). The export apparatus is composed by the C ring (a measure cup), and C rod (the export gate). These substructures are under vigorous investigation at the moment.

Nearly 50 genes are involved in the assembly of peritrichous flagella in Salmonella typhimurium. Those genes are divided into three classes and their expression is hierarchically controlled; class 1 (the master genes) controls class 2 (genes encoding the hook-basal body components), and a sigma factor (FliA) and the anti-sigma factor (FlgM) of the class 2 control the expression of class 3 (flagellin, chemotaxis proteins and others).

Transport of flagellar proteins from the chromosome to the export apparatus is still ambiguous. In order to elucidate the transport mechanism, localization of flagellar proteins in the cytoplasm was examined by a combination of several techniques: quick-freezing of cells at the liquid helium temperature, thin-sectioning of fixed cells, and immuno-electron microscopy. The results will be presented at the meeting.

Characterisation of PcrH in *Pseudomonas aeruginosa* type III secretion, a regulatory deficient translocator class chaperone

Jeanette E. Bröms, Anna-Lena Forslund, Ake Forsberg and Matthew S. Francis

Pseudomonas aeruginosa harbors a type III secretion system to translocate anti-host effectors into an infected eukaryotic cell. In this essential virulence strategy, we highlight PcrH as a key type III secretion component. In the absence of PcrH, *P. aeruginosa* was translocation deficient due to a specific reduction in pre-secretory stability and subsequent secretion of PopB and PopD. These proteins are essential for the translocation process(1-3). PcrH exerts this chaperone function by binding directly to PopB and PopD. Consistent with the genetic relatedness of PcrH with LcrH of pathogenic Yersinia, these proteins were functionally interchangeable with respect to their ability to complement the translocation defect associated with either a lcrH or pcrH null mutant respectively. Thus, the translocator class of chaperones performs a critical function in ensuring the assembly of a translocation competent type III secreton. Finally, unlike the regulatory roles of other translocator class chaperones including LcrH, SicA of Salmonella enterica and IpgC of *Shigella sp.(*4-5), in vitro regulation of *P. aeruginosa* type III secretion does not involve PcrH.

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Enteropathogenic *Escherichia coli* Type III translocation system

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Enteropathogenic *Escherichia coli* (EPEC) is an important cause of infantile-diarrhoea in developing countries. The hallmark of EPEC infection is induction of a distinct attaching and effacing (A/E) intestinal lesions, which is characterised by local destruction of brush border microvilli and intimate bacterial adhesion to the host cell plasma membrane. Like many Gram-negative pathogens EPEC relies on its type III secretion system to translocate effector proteins which subvert normal cellular function for the benefit of the extra cellular bacterium. (Frankel et al., 1998). The EPEC type III secretion apparatus is unique as it has a filamentous extension to the basic needle complex; EspA is the main or only constitute of these hollow filamentous structures (Daniell et al., 2001 and 2003). EspA filaments are essential for A/E lesion formation as an *espA* mutant secretes, but is unable to translocate, the effector proteins (Knutton et al., 1998; Shaw et al., 2001). Although EspA filaments are not formed in the absence of the translocator protein EspD, the role EspD plays during filament assembly is not known. We are investigating the mechanism of EspA filament assembly and function; the results of this investigation will be presented in this poster.

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Assessment of effector delivery in Yersinia LcrH chaperone mutants impaired for cognate translocator stability and secretion

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To establish an infection, *Yersinia pseudotuberculosis* utilizes a plasmid-encoded type III secretion machine that permits the translocation of several anti-host effectors into the cytosol of target eukaryotic cells (1). The YopD and YopB translocator proteins are essential for this process (2,3). Pre-secretory stabilization of the translocators is mediated by an interaction with the cognate chaperone, LcrH (4,5). We investigated whether site-directed mutants of LcrH impaired the translocation of anti-host effectors into infected cells. We specifically isolated LcrH mutants that displayed a reduced ability to bind YopB and YopD by the yeast two-hybrid assay. When introduced in cis onto the virulence plasmid of *Y. pseudotuberculosis*, these mutants were unable to maintain stableYopB or YopD, nor promote their efficient secretion. We considered that these mutants would also be defective for effector translocation, yet were surprised to observe that they were as efficient as wild type Yersinia for delivery of effectors into HeLa cell monolayers. Thus, a minimal level of secreted translocator protein is apparently all that is required for afunctional type III translocon.

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Crystal structure of the $HrcQ_B$-C protein from *Pseudomonas syringae* Hrp secretion system

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$HrcQ_B$ is a low-molecular weight (14 kDa) hydrophilic protein which belong to the *Pseudomonas syringae* pv. phaseolicola type III secretion machinery. It is assumed to have a structural role on the putative basal body of the Hrp apparatus. The full-length protein is sensitive to proteolysis and has resisted all crystallization attempts.

However, its C-terminal domain that comprises 84 amino acids and has a molecular weight of 9 kDa ($HrcQ_B$-C) is proteolytically stable and it has readily been crystallized from MPD using the hanging drop vapour diffusion method. This fragment represents the conserved domain among the homologous proteins of other plant and animal pathogenic bacteria and the flagellum and retains the ability of the full-length $HrcQ_B$ to interact with Hrp proteins. The crystals belong to space group $P2_1$, with unit cell parameters a=52.9, b=27.7, c=99.0 Å and beta=99.8°.

The crystal structure was determined by Multiwavelength Anomalous Dispersion (MAD) and refined to 2.3Å resolution. $HrcQ_B$-C is an elongated, gently curved homotetramer with approximate dimensions of 25 x 30 x 90 $Å^3$. The four monomers assemble into two tightly bound homodimers which are packed together to form a dimer of dimers. The dimers associate with each other in such a way that the angle between their major axes of inertia is approximately 120 degrees. The secondary structure of each monomer consists of five β-strands and one, short helix. Two monomers fold together in a symmetrical manner to form a compact and intertwined dimeric structure that comprises two six-stranded antiparallel β-sheets bridged through a long β-ribbon.

A three dimensional cluster analysis of sequence conservation patterns reveals two clusters of residues potentially involved in protein-protein interactions.

Secretion defective variants of pullulanase from *Klebsiella oxytoca*

Olivera Francetic, Anthony Pugsley

Pullulanase (PulA) from *Klebsiella oxytoca* is a 116 kD lipoprotein of the isoamylase family that is secreted to the bacterial cell surface via the type II secretion pathway. A nonacylated variant of PulA was made by fusing the mature part of the protein to the signal peptide (sp) of the pectate lyase PelB from *Erwinia chrysanthemi*. The PelBsp-PulA was efficiently secreted in *Escherichia coli* expressing the complete set of genes encoding the pullulanase secretion machinery (the Pul secreton). Mutational analysis was performed on this PulA variant to define the region of the protein required to promote its secretion. Previously, two regions of PulA, region A (amino acid residues 1 to 78) and region B (residues 735 to 814) were identified as parts of the secretion signal. A series of in-frame deletions, insertions and fusions to different reporter proteins in the context of the nonacylated PulA was constructed to validate these findings. In particular, transposon mutagenesis was used to generate PelBsp-PulA variants containing 24 amino acid peptide insertions at random positions. Four independent insertions were found that completely abolished secretion. They clustered in the region between amino acid residues 234 and 323. This region of PulA, designated C, could contain a part of the PulA secretion signal or be important for the presentation of the secretion signal to the Pul secreton. A deletion of the *pulA* fragment encoding residues 288 to 430 (including region C) abolished the secretion of PulA without affecting its stability. Staphylococcal nuclease NucB reporter protein was secreted when fused to PulA after residue 428, but not when fused after residue 70 (region A) or to PelBsp-A-B. Determination of the PulA protein structure in conjunction with fine mutational analysis of will be required to define the pullulanase secretion signal.

VacA β-barrel mediates secretion of EspC

Louise Arnold, Thomas Baldwin and Kim R Hardie

Characterization of the IgA protease secreted from *Neisseria meningitides* heralded the discovery of a family of autotransporter proteins which include virulence factors of (i) pathogenic *E. coli* such as EspC (secreted by some strains of Enteropathogenic *E. coli*, (EPEC) and PET (secreted by Enteroaggregative *E. coli*, (EAggEC), and (ii) the vacuolating toxin (VacA) secreted by the gastric pathogen, *H. pylori*.

Autotransporters are translated as large precursor polypeptides in the cytoplasm. An N-terminal targetting signal directs the translocation across the cytoplasmic membrane and is cleaved in the process. This is proposed to occur with the aid of the Sec machinery, and subsequently the C-terminal domain of the polypeptide inserts into the outermembrane forming a β-barrel through which the central passenger domain is transported. Once at the cell surface, the mature passenger domain is released following cleavage which is achieved either by cell associated proteases, or via the autoproteolytic activity of certain autotransporters e.g. IgA protease.

This study has demonstrated that the β-barrel domains of EspC, PET and VacA are functionally interchangable. Moreover, EspC, PET and hybrid autotransporters could successfully translocate across the two membranes of laboratory adapted *E. coli* strain MG1655, indicating that no accessory factors specific to the original pathogenic host are required for translocation.

Determining whether Tat signal peptides show specificity towards their cognate passenger proteins

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A characteristic feature of proteins exported through the bacterial Tat pathway is that they are exported in their fully folded, active form. In many cases this involves the binding of metal cofactors, and often partner subunits before the export event. Thus it is likely that the cell suppresses the export of the substrate protein until it is fully assembled and active. The signal peptide has been implicated in playing an important role in the active assembly of its cognate redox enzyme (Sambasivarao et al., 2000; Blaudeck et al., 2001). Clearly these observations have important implications for the export of heterologous proteins.

In our efforts to identify the most effective Tat targeting signal for the export of foreign proteins, we have undertaken a study to investigate the role of the signal peptide in targeting and assembly. To achieve this aim, we have precisely replaced the signal sequence of the single subunit molybdoenzyme TMAO reductase with that of the small subunit of Hyb0, which directs export of the heterodimeric nickel iron protein Hydrogenase 2. The signal sequence replacement has been recombined onto the chromosome of *E. coli* at the *tor* locus so that we can confidently study the effect of this change on the export of TMAO reductase. We have also made the complementary replacement of the HybO signal sequence with that of TorA and recombined this into the chromosome at the *hyb* locus. Our results on the assembly and export of TMAO reductase, and of Hydrogenase 2 in these strains will be reported.

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Role of coupling proteins in bacterial conjugation

Matxalen Llosa, Sandra Zunzunegui, and Fernando de la Cruz

Bacterial conjugation is a complex process that involves DNA processing and protein/DNA secretion. In Gram-negative bacteria, a set of proteins constitute a type IV secretion system (T4SS) that forms a transmembrane channel. The two-step model for bacterial conjugation (1) postulates that T4SS might be needed to transport a pilot protein, the relaxase, that would drive the displaced replicating DNA strand through the secretion pore. A DNA transporter would then pump out the rest of the DNA molecule. Conjugative coupling proteins are proposed to play a role in connecting the relaxosome to a T4SS during bacterial conjugation, and are also putative DNA transporters with NTP and DNA binding abilities. We have addressed an analysis of the protein-protein interactions of coupling proteins with both relaxosome and T4SS components in several conjugative systems (2). We present biochemical evidence indicating that the prototype coupling protein, TrwB, interacts with TrwA and TrwC, the components of R388 relaxosome. The coupling protein of the related IncN plasmid pKM101 did not interact with TrwA or TrwC, suggesting a highly specific interaction with the relaxosome. By using the bacterial two-hybrid system, a strong interaction was detected between TrwB and TrwE, a core component of conjugative T4SS. Coupling proteins from three different conjugation systems were shown to interact with both their cognate TrwE-like component and with the heterologous ones, suggesting that this interaction is less specific. Mating experiments among the three systems confirmed that relaxosome components need their cognate coupling protein for transfer, while T4SSs are interchangeable to a certain extent. Moreover, there is a correlation between the strength of the interaction seen by two-hybrid analysis and the efficiency of transfer.

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Membrane restructuring by *Bordetella pertussis* adenylate cyclase-toxin, a member of the RTX toxin family

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Adenylate cyclase toxin (ACT) is secreted by *Bordetella pertussis*, the bacterium causing whooping cough. ACT is a member of the RTX (repeats in toxin) family of toxins and, like others members in the family, it may bind cell membranes and cause disruption of the permeability barrier, leading to efflux of cell contents. The present paper summarizes studies performed on cell and model membranes, with the aim of understanding the mechanism of toxin insertion and membrane restructuring leading to release of contents. ACT does not necessarily require a protein receptor to bind the membrane bilayer, and this may explain its broad range of host-cell types. In fact, red blood cells and liposomes (large unilamellar vesicles) display a similar sensitivity towards ACT. Toxin effects are however suppressed by poly (ethylene glycol) grafted onto the membrane surface. Ca2+ is not required for ACT insertion into a lipid monolayer at the air-water interface, but efflux occurs only in the presence of millimolar Ca2+. Release of aqueous vesicular contents is enhanced by the presence of negative-curvature lipids (phosphatidylethanolamine, cholesterol), and inhibited by lipids favouring positive curvature (lysophosphatidylcholine). Moreover, ACT influences the lamellar-hexagonal phase transition of phosphatidylethanolamine, decreasing its cooperativity. These results strongly suggest that ACT-induced efflux is mediated by transient non-lamellar lipid structures. This is confirmed by the experimental observation that efflux is accompanied by transbilayer ("flip-flop") lipid motion in the same time scale.

Secretion and virulence of *Pseudomonas aeruginosa* mutants overexpressing multidrug efflux pumps

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Pseudomonas aeruginosa is one of the most relevant opportunistic pathogens worldwide. The incidence of *P. aeruginosa* infections at hospitals ranks at first or second position depending on the geographical localization. One of the most cumbersome characteristics of *P. aeruginosa* resides in its intrinsically high resistance to antibiotics. *P. aeruginosa* intrinsic antibiotic resistance is mainly due to the presence of several multidrug resistance (MDR) efflux pumps in its genome [1]. Expession of those pumps is usually repressed, at least under laboratory growing conditions. However, de-repressed mutants are easily selected both *in vitro* [2] and *in vivo* [3]. It has been largely assumed that acquisition of an antibiotic resistance phenotype might produce a reduction on bacterial fitness [4]. This reduction might be accompanied by a reduction in the virulence properties of bacteria. Work from our laboratory [5] and others [6] has demonstrated that overproduction of MDR pumps might be associated with a reduction on *P. aeruginosa* virulence.

It is well known that bacterial secreted compounds have a relevant role on virulence. In the case of *P. aeruginosa*, the role of Type III secreted proteins [7] and other compounds such as pyoverdine or piocyanin on the virulence of this bacterial pathogen has been well established. We wanted thus to analyse the effect of overproduction of MDR determinants on *P. aeruginosa* secretion. To that goal, single-step spontaneous mutants overexpressing each of the four MDR pumps so far biochemically characterized in *P. aeruginosa* were obtained. Expression of the pumps was analyzed by RT-PCR so that each mutant overexpressed just one pump. The proteins secreted by wild-type and MDR mutant strains at different times of incubation were analyzed by 1-D SDS-PAGE and MALDI-TOF mass spectrometry. The analysis was also performed under conditions that stimulate Type III secretion. Among the observed changes, the most relevant one was a reduction on the secretion of some Type III-secreted effectors by the mutant overexpressing MexEF-OprN. A reduction on the secretion of some other virulence factors was also observed for thismutant.

The virulence of MDR-overproducing mutants was tested in the non-mammalian model *Dyctiostelium discoideum* [6]. Data will be presented on the effect of overproduction of MDR pumps on *P. aeruginosa* secretion and virulence.

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The biochemical characterisation of *E.coli* Tat protein translocases

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The *Escherichia coli* Tat apparatus is a protein translocation system that serves to export folded proteins across the cytoplasmic membrane. The integral membrane proteins TatA, TatB and TatC are essential components of this pathway. Substrate proteins are directed to the Tat apparatus by specialized N-terminal signal peptides bearing a consensus twin-arginine sequence motif. Overexpression of the tatABCDE genes yielded a protein complex predominantly composed of a TatBC unit with a small amount of TatA, when purified using a C-terminal his-tag on TatC. Analysis of Tat complexes at native expression levels in *E. coli* membranes indicated that only a small amount of TatA co-purified with the TatBC unit indicating that the purified overexpressed Tat(A)BC complex was not an artefact. The Tat(A)BC complex was determined by analytical ultracentrifugation to a have an approximate molecular mass of 613 kDa and contained less than 1 mol of phospholipid per mol of purified complex. Furthermore, blue native PAGE techniques demonstrated that the Tat(A)BC complex was distinct from a homooligomeric TatA complex present in *E. coli* cytoplasmic membranes. Electron microscopy of the complex will also be presented.

Structural requirements for FimH recognition

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Type 1 fimbriae (or pili) are thin protein polymers found in the surface of *E. coli* involved in the adhesion of bacteria to different epithelia. They are composed by a major subunit (FimA) and different minor protein subunits, being one of them the adhesin (FimH). Fimbriae assembly requires a periplasmic chaperone (FimC), which binds the subunits, and an outer membrane (OM) usher (FimD) for their ordered polymerization and secretion.

In this communication we analyze the structural features of FimH adhesin required for its recognition by FimC and FimD. FimH is composed of two immunoglobulin (Ig)-like domains connected by a short linker. We have generated a collection of FimH derivatives with different linker lengths that replace their N-terminal adhesin domains by Ig domains derived from antibodies (e.g. human VL or VHH camelbodies). We analyzed the recognition of these FimH-variants by FimC / FimD and their secretion and assembly in fimbriae.

Study of the role of cholesterol oxidase in the virulence of the intracellular pathogen *Rhodococcus equi*

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The virulence mechanisms of the facultative intracellular parasite *Rhodococcus equi* remain largely unknown. Among the candidate virulence factors of this pathogenic actinomycete is a secreted colesterol oxidase, a membrane-damaging toxin. The gene encoding this enzyme, *choE*, was previously identified and characterized in our laboratory (1). In order to analyse the role of cholesterol oxidase in the virulence of *R. equi* a choE-deletion mutant was generated in the strain *R. equi* 103. This is the first unmarked mutant obtained in *R. equi* and it constitutes the basis for the generation of attenuated strains potentially useful as vaccines to prevent pneumonia caused by *R. equi*. To investigate the role of cholesterol oxidase in virulence we performed invasion and proliferation assays in both murine macrophages J774A-1 and CACO-2 epithelial cells. A murine animal model was also used to assert the role of choE in *R. equi* virulence.

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Structure function relationships in the SecDFyajC complex

Nico Nouwen and Arnold J.M. Driessen

In bacteria, transport of the majority of secretory proteins across the cytoplasmic membrane and insertion of most membrane proteins into the cytoplasmic membrane is catalysed by a multisubunit enzyme complex called 'translocase'. The *Escherichia coli* translocase is composed of a peripheral ATPase, SecA and a heterotrimeric integral membrane domain with SecY, SecE and SecG as subunits. SecD, SecF and YajC form another heterotrimeric complex that can associate with the SecYEG complex. Despite the progress made in recent years towards the understanding of the translocation mechanism and structural features of the SecYEG complex, the role of the SecDFyajC complex in the translocation process remains unclear.

A remarkable feature of SecD and SecF is that they both contain a very large first periplasmic loop (P1) that compromises about one third of the total size of the protein. To study the role of this periplasmic loop in SecDF-functioning we constructed SecD and SecF mutants with deletions in this loop. All constructed mutants showed a cold-sensitive growth phenotype and isolated inner membrane vesicles from these strains show a reduced translocation *in vitro*. Whereas some results could be contributed to a reduced expression of the deletion mutant other mutants were normally expressed and able to form a stable SecDFyajC complex. Using the latter mutants we try to address which step in the translocation process is affected.

Involvement of the TAT (Twin Arginine Translocation) protein export system from *Rhizobium leguminosarum* in both free living and symbiotic life styles

L. Rey and J. Palacios

Up to 20% of the proteins synthesized in bacterial cells are exported out of the cytoplasm. Some periplasmic proteins acquire partial of full conformation in the cytoplasm, usually by binding cofactors in this cell compartment. Translocation of these proteins cannot proceed through the general secretion (Sec) pathway, but the TAT (Twin-Arginine Translocation) pathway, a system capable of exporting proteins in folded conformation (1), is used instead. Proteins exported via this system harbor a characteristic signal peptide with a double arginine motif that designates the system. The *E. coli* TAT translocon contains four proteins, namely TatA/TatE, TatB and TatC. However, a general model showing the functioning of such translocon has not been elucidated yet.

We are currently studying the potential role of the TAT system in legume endosymbiotic bacteria. To this end, a *Rhizobium leguminosarum bv viciae* gene cluster encoding TatA, TatB and TatC proteins was cloned and sequenced, and a tatBC mutant was generated. Cultured cells of the TAT-deficient mutant were impaired in ubiquinone-cytochrome c oxidoreductase activity and also lacked cytochrome c-dependent respiration. Consistently with that, a Tat-type signal peptide was identified in the Rieske component of *R. leguminosarum* cytochrome bc1 complex. The same mutant was unable to fix nitrogen in symbiosis with pea plants (2). Since cytochrome bc1 complex, a critical branchoint for cytochrome c-dependent respiration in *R. leguminosarum*, is essential for symbiosis (3), these data suggested that the inability to synthesize such complex might be the reason for the Fix-negative phenotype of the mutant strain. However, structural analysis of nodules induced by GFP-tagged *R. leguminosarum* derivatives revealed that the TAT mutation results in the impairment of the ability to colonize the nodule central region, a trait that is not affected in a cytochrome bc1 mutant. This result indicates that the Tat mutant was also affected in early steps of the symbiotic process. Analysis of outer cell components showed that the TAT-deficient mutant synthesized an altered form of EPS. Candidate proteins involved in Tat-dependent EPS synthesis are currently under examination.

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Streptomyces lividans SRP is involved in protein secretion

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The bacterial version of the mammalian signal recognition particle (SRP) is well conserved and essential to all known bacteria. SRP is a ribonucleoprotein, which recognises signal peptides of proteins that are targeted to the membrane in Gram-negative bacteria, whereas in Gram-positive bacteria the SRP transports secretory proteins as well. This feature emphasizes the major importance of the SRP in Gram-positive bacteria and enhances the interest on studying its function.

The genes for the Gram-positive soil bacterium *Streptomyces lividans* TK21 SRP components (Ffh, scRNA and FtsY) have been cloned and characterized. *S. lividans* SRP consists of Ffh, a homologue of the mammalian SRP54 protein, and scRNA, which is a small size RNA of 82 nt in length. FtsY resembles the mammalian SRP receptor. Co-immunoprecipitation studies confirmed that Ffh and scRNA are probably the only two components of the *S. lividans* SRP. To test the secretory function of SRP, immunoprecipitation studies using anti-agarase, anti-alpha amylase or anti-Ffh sera clearly showed co-immunoprecipitation of these proteins with Ffh, suggesting that the SRP is involved in targeting secretory proteins.

Current work is focussed on determining the interactions of FtsY with other components of the secretory system.

The TatA component of the *Escherichia coli* twin arginine translocase pathway

Ida Porcelli

The *Escherichia coli* Tat apparatus is a multiprotein system that serves to export folded proteins across the inner membrane. Proteins translocated via the Tat pathway bear a characteristic signal peptide with a twin-arginine consensus motif. Essential components of this system are the integral membrane proteins TatA, TatB, and TatC. On a molar basis, TatA is the major component of the pathway. TatA is an integral membrane protein of 89 amino acids with a predicted transmembrane alpha-helix at the amino-terminus of the protein followed by a segment containing a predicted amphipatic alpha-helix. Chemical cross-linking studies showed that TatA froms at least tetrameric homooligomers in the cytoplasmic membrane. Non-denaturing purification of TatA yielded a homooligmeric complex of 460kDa. Deletion of the amino-terminal membrane-anchoring domain resulted in the loss of homooligmer formation. Circular dichroism spectroscopy has indicated that the transmembrane domain has importance in the correct folding of TatA. An expression construct, lacking the transmembrane segment, produced TatA that was largely unstructured in aqueous solutions but membrane association of the protein induced alpha-helix formation. Protease accessibility experiments have demonstrated that the extramembranous region of TatA is located at the cytoplasmic side of the inner membrane.

The role of harpins in the translocation of effectors into plant cells by the *Pseudomonas syringae* type III secretion system

Adela R. Ramos, Lisa Shechter, and Alan Collmer

Pseudomonas syringae pv. tomato DC3000 causes bacterial speck disease on tomato. The type III secretion system (TTSS) is essential to the virulence of *P. syringae*. A suite of genes encodes the central TTSS apparatus, which is conserved among bacterial plant and animal pathogens that have the TTSS. In *P. syringae* these genes are located in the hrp/hrc cluster (hypersensitive response and pathogenicity/ hrp conserved) within a pathogenicity island (1). In this cluster, there is also a set of genes encoding proteins unique to plant pathogens, and these are likely to have a role in promoting penetration of the thick (200 nm) plant cell wall (2). Two of these proteins thought to help in the extracellular translocation process are the HrpZ and HrpW harpins. Harpins are glycine-rich, acidic, cysteine-lacking, heat stable proteins that can elicit plant defenses when exogenously applied. HrpZ is encoded within the *hrp/hrc* cluster, whereas HrpW is encoded elsewhere in the pathogenicity island. The role of harpins in pathogenesis remains puzzling (3-6). Here, we searched the recently sequenced *P. syringae* pv. tomato DC3000 genome, and found other potential harpin candidates scattered throughout the chromosome. These harpin candidates also elcit plant defenses when infltrated into plants, and they also share an important similarity with HrpW: they have two domains, one harpin-like domain and one domain with homologies to enzymes that modify plant cell walls. HrpW shares similarity with pectic lyases, as does HopPmaHPto, while HopPtoP has homology to transglycosylases. These findings support our hypothesis that harpins aid the TTSS in translocation of effectors by modifying the plant cell wall. The numerous harpins in the genome of *P. syringae* necessitates the use of a reductionist system to analyze harpin function. In order to study the effect of a single harpin, we have introduced a cosmid containing an *hrp/hrc* cluster that includes *hrpZ*, as well as one without *hrpZ*, into a nonpathogenic bacterium, *Pseudomonas fluorescens* 55. Each strain also carries a construct with an effector gene, avrPto, fused to *cya*, a reporter gene. Without *hrpZ*, the bacterium is significantly impaired in its ability to translocate AvrPto into plant cells. Complementation of the translocation defect with different harpins is currently being investigated. These experiments should contribute to our understanding of harpins, which are unique to plant pathogen TTSS.

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Biofilm formation of pathogenic *E. coli* isolates

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A significant number of infectious diseases caused by the versatile opportunistic pathogen *Escherichia coli* are initiated by bacterial adhesion to the surfaces of the human genitourinary, gastrointestinal or respiratory tracts. Although the adhesion mechanisms utilized by pathogenic *E. coli* have been extensively studied and are crucial for the infection process, they are only part of the story. Several post-adhesion events are required for bacterial cells to establish themselves and to initiate infection that may include e.g. up-regulation of expression of virulence factors. A certain threshold number of adhered bacterial cells that withstands host defence – either attained by continuous bacterial adhesion or by cell proliferation on the surface – appears in many cases to be a prerequisite for a successful infection. Several in situ studies suggest that as in most natural bacterial surface-associated communities (biofilms), many adhered bacterial cells at a given infected site do not have a direct cell-surface contact but are rather adhered to other *E. coli* cells. We therefore tested 396 *E. coli* isolates in *in vitro* biofilm models to characterize the conditions that promote efficient cell-cell adhesion on abiotic surfaces. Subsequent molecular approaches were applied to dissect the genetic repertoire that allows pathogenic *E. coli* strains to stick to each other and to withstand strong shear forces present at many host sites.

Role of bacterial resistance to inhibitory substances in *Erwinia chrysanthemi* pathogenicity

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Most phytopathogenic bacteria are specialized in colonizing the plant apoplast. This particular niche is relatively nutrient-poor and it is laden with antimicrobial substances. Moreover, plant apolast is acidic, with pH ranging from 4.0 to 6.5, due to the presence of organic acids and the extrusion of protons from near-by cells. These pH values pose a significant barrier for bacterial development.

Erwinia chrysanthemi belong to the enterobacteriaceae family, and is a causal agent of the "soft-rot" disease in many important crops worldwide. The pathogenic behaviour of this bacterium is characterized by a quick necrosis of parenchymatic tissues, due to the secretion of pectolytic enzymes which degrade the primary cell wall. It is known that this process leads to plant cell death, release of cell contents to the apoplastic space and alkalinization of the apoplast. In turn, this improves the condition for bacterial growth and the activity of pectolytic enzymes, opening a process that eventually produces the maceration of large areas. Our long-term goal is to understand the mechanisms that enable *E. chrysanthemi* to survive in the harsh conditions prevalent in the plant apoplast. In particular, we have focused in the role of bacterial resistance against antimicrobial peptides (1,2,3), to active oxygen species (4), as well as the importance of competition with endophytic bacteria (5). Presently, since the *E. chrysanthemi* genome is already available (http://www.tigr.org), we are using this information to generate mutations in genes possibly involved in aspects relevant for pathogenicity; mainly, a) to evaluate the role of acidic pH as a barrier for *E. chrysanthemi*, and b) to study the role of bacterial transporters involved in the efflux of a high range of toxic substances.

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Posttranslational processing of the xylanase Xys1L from *Streptomyces halstedii* JM8 is carried out by secreted serine proteases

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The xylanase Xys1L from *Streptomyces halstedii* JM8 is processed extracellularly, to produce a protein of 33.7 kDa, Xys1S that retains catalytic activity but not its cellulose-binding capacity (Ruiz Arribas et al. 1997). We have demonstrated that several serine proteases isolated from *Streptomyces* have the ability to process the xylanase Xys1L. The genes of two of these extracellular serine proteases were cloned from *S. lividans* 66 (a strain commonly used as a host for protein secretion), sequenced, and overexpressed in *S. lividans*, and both purified proteases were able to process Xys1L *in vitro*. Three other previously reported *Streptomyces* purified serine proteases, SAM-P20, SAM-P26 and SAM-P45- also processed Xys1L *in vitro*. The involvement of serine proteases in xylanase processing-degradation *in vivo* was demonstrated by co-expression of the xylanase gene (*xysA*) and the gene coding for the serine protease inhibitor (SLPI) from *S. lividans*. Co-expression prevented processing and degradation of Xys1L and resulted in a three-fold increase in the xylanase activity present in the culture supernatant. SpB and SpC have also the capacity to process other secreted proteins such as p40, a cellulose-binding protein from *S. halstedii* JM8, but do not have any clear effect on other secreted proteins such as amylase (Amy) from *S. griseus* and xylanase Xys30 from *S. avermitilis* (Fernandez Abalos et al 2003).

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Use of the Cya reporter to investigate translocation of effector proteins into plant cells by the Hrp secretion system of *Pseudomonas syringae*

Lisa M. Schechter, Katherine A. Roberts, Yashitola Jamir, James R. Alfano, and Alan Collmer

Pseudomonas syringae pathovar tomato strain DC3000 is a pathogen of tomato and Arabidopsis. The hrp-encoded type III secretion system, which injects bacterial effector proteins (primarily called Hop or Avr proteins) into plant cells, is required for virulence. A variety of genetic and bioinformatic methods have recently been employed to identify many Hrp-secreted proteins in DC3000, as well as even more potential Hop candidates. In addition to being regulated by hrpL, most hop genes encode proteins with N-termini that possess several characteristic features, including: (i) a high percentage of Ser residues, (ii) an aliphatic amino acid (Ile, Leu, or Val) or Pro in the third or fourth residues, and (iii) a lack of negatively charged amino acids (Asp or Glu) within the first 12 residues. In this study, a calmodulin-dependent adenylate cyclase (Cya) reporter system was optimized to study *P. syringae* Hrp-mediated translocation of the known effector protein, AvrPto. AvrPto-Cya induces cAMP accumulation in both tomato and tobacco plants when translocated by either *P. syringae* or *P. fluorescens* expressing a *P. syringae* Hrp system. Analyses of truncated AvrPto and AvrB proteins fused to Cya confirm that N-terminal sequences are required for the translocation of these effectors into plant cells. The translocation of several DC3000 candidate Hop proteins was also examined using Cya as a reporter, which led to the identification of three new intact Hop proteins, named HopPtoQ, HopPtoT1, and HopPtoV, as well as two truncated Hop proteins, named HopPtoS4'(N) and HopPtoW'(N). We also confirm that HopPtoK, HopPtoC, and AvrPphEPto are translocated into plant cells. Although most of the newly identified Hops possess N-termini that share the same features as known Hops, HopPtoV contains none of these characteristics. Our results indicate that Cya will be a useful reporter for exploring multiple aspects of the Hrp system in *P. syringae*.

BAPs, a novel family of surface proteins involved in bacterial biofilm formation process

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Genetic analysis of biofilm formation process of different bacterial species has identified a novel family of proteins, named as BAPs (Biofilm Associated Proteins), which are important for biofilm formation process of both Gram-positive and Gram-negative bacteria. Members of this family have been described in *Staphylococcus aureus* (Bap), *S. epidermidis* (Bhp), *Enterococcus faecalis* (Esp), *Burkholderia cepacia* (Bap), *Pseudomonas putida* (*mus20*), *Salmonella typhimurium* (*stm*2689). All members of BAP family share the following characteristics: (i) high-molecular-weight; (ii) signal sequence for extracellular secretion; and (iii) a core domain of repeats, which number varies among different isolates. Disruption of the *bap* gene resulted in mutant strains unable to produce biofilm. In contrast, in-frame deletion of the repeats region did not affect the biofilm formation capacity. For those that have been studied, deletion of *bap* decreased surface hidrophobicity, suggesting changes in the cell surface properties. Analysis of primary structure of BAPs members revealed the presence of potential EF-hand calcium-binding motifs. In the case of *S. aureus*, biofilm formation capacity of Bap-positive strains is affected by the presence of millimolar amounts of calcium. Site directed mutagenesis of the putative EF-hand motifs resulted in a strain capable of forming biofilm but whose biofilm development was not affected by the presence of calcium. We have no evidence for either dimerization of BAPs or direct interaction between BAPs and the exopolysaccharide matrix of the biofilm or direct ligand binding activity of BAPs to host receptors. Further studies are urgent to either exclude or confirm any of these possibilities.

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*8 **Workshop on the Diversity of the Immunoglobulin Superfamily.**
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*15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
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*23 **Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.**
Organizers: C. M. Thomas, E. M. H. Willington, M. Espinosa and R. Díaz Orejas.

*24 **Workshop on Deterioration, Stability and Regeneration of the Brain During Normal Aging.**
Organizers: P. D. Coleman, F. Mora and M. Nieto-Sampedro.

*25 **Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.**
Organizers: J. J. Bujarski, S. Schlesinger and J. Romero.

26 **Workshop on Cellular Interactions in the Early Development of the Nervous System of Drosophila.**
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32 **Workshop on Chromatin Structure and Gene Expression.**
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35 **Workshop on Signal Transduction Pathways Essential for Yeast Morphogenesis and Cell Integrity.**
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*44 **Workshop on Selective Gene Activation by Cell Type Specific Transcription Factors.**
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*49 **Workshop on Transcriptional Regulation at a Distance.**
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50 **Workshop on From Transcript to Protein: mRNA Processing, Transport and Translation.**
Organizers: I. W. Mattaj, J. Ortín and J. Valcárcel.

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Organizers: M. Barbacid and D. Pulido.

66 **Workshop on 100th Meeting: Biology at the Edge of the Next Century.**
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67 **Workshop on Membrane Fusion.**
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91 **Workshop on Eukaryotic Antibiotic Peptides.**
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93 **Workshop on Cell Cycle Regulation and Cytoskeleton in Plants.**
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94 **Workshop on Mechanisms of Homologous Recombination and Genetic Rearrangements.**
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148 **Workshop on Membranes, Trafficking and Signalling during Animal Development.**
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