THE *YERSINIA* YSC–YOP 'TYPE III' WEAPONRY

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'Type III secretion' — the mechanism by which some pathogenic bacteria inject proteins straight into the cytosol of eukaryotic cells to 'anaesthetize' or 'enslave' them — was discovered in 1994. Important progress has been made in this area during the past few years: the bacterial organelles responsible for this secretion — called 'injectisomes' — have been visualized, the structures of some of the bacterial protein 'effectors' have been determined, and considerable progress has been made in understanding the intracellular action of the effectors. Type III secretion is key to the pathogenesis of bacteria from the *Yersinia* genus.

EXOTOXINS
Bacterial protein toxins that are secreted by pathogenic bacteria and that contribute to infectious disease.

MACROPHAGES
Long-lived bone-marrow-derived cells that are central to the host defence against microbes. Their main functions are phagocytosis, antigen-presentation and the release of inflammatory cytokines.

POLYMORPHONUCLEAR LEUKOCYTES
Short-lived bone-marrow-derived cells with high motility and phagocytic capacities.

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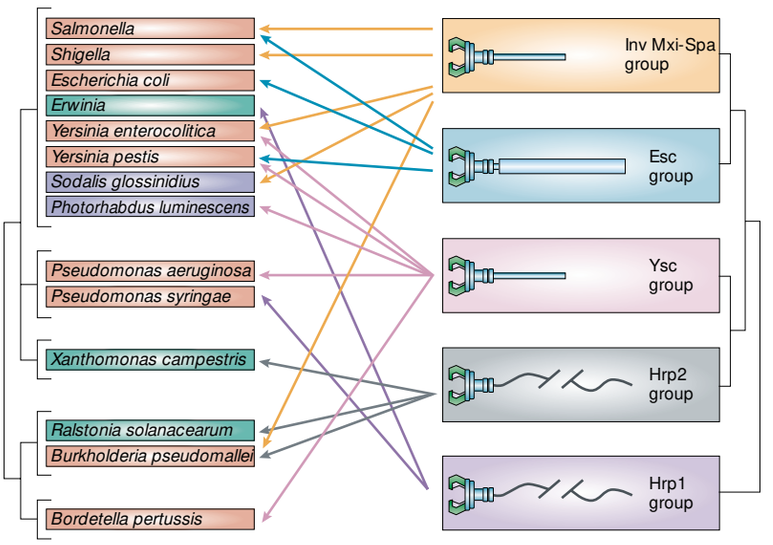
Type III secretion (TTS) is a mechanism by which Gram-negative bacteria that are either extracellular or localized in phagosomes communicate with eukaryotic cells by injecting bacterial proteins across cellular membranes into the cytosol of these cells. These bacterial proteins act as powerful 'effectors' and take control of the host cells by hijacking their intracellular machinery[1,2]. More than 20 TTS systems have now been discovered in animal, plant and even insect pathogens, and the list remains open (BOX 1). The target cells respond to this 'injection' in different — or even completely opposite — ways depending on the system that is involved. Although the *Yersinia* Ysc–Yop system that is described here essentially paralyses specialized phagocytes, some others, such as the *Shigella flexneri* Mxi–Spa or the *Salmonella enterica* SPI-1 systems, trigger phagocytosis by cells that are normally non-phagocytic (for reviews on TTS systems in pathogenesis see REFS 3–6).

For animal pathogens, we can estimate that each of the systems injects between 5 and 10 effectors, and so the complete array of TTS effectors in this group must consist of more than 100 proteins. These TTS effectors take control of the animal cell in an exquisitely refined way, and they generally have more sequence homology to eukaryotic proteins than to bacterial proteins. This indicates that they originate from genetic material borrowed from the host during evolution, which implies that eukaryotic counterparts could be identified for most of them, and that their identification might help cell biologists to fill gaps in, or to complete, known regulatory circuits. Therefore, type III effectors are making their way in the field of molecular cell biology, as the bacterial EXOTOXINS and several viral proteins did some time ago.

Rather than reviewing the exploding field of TTS, this article illustrates the phenomenon by describing the Ysc–Yop system of *Yersinia* (BOX 2), which is one of the best understood. Our understanding of this system, from which the TTS concept emerged[1,2,7], has now reached a stage where many facts can be integrated into a concept that accounts quite well for the in-host lifestyle of these pathogens. The plasmid-encoded Ysc–Yop TTS system allows extracellular *Yersinia* that is docked at the surface of cells of the immune system to deliver Yop effectors into the cytosol of these cells. These effector Yops disturb the dynamics of the cytoskeleton and block phagocytosis by MACROPHAGES and POLYMORPHONUCLEAR LEUKOCYTES (PMNs)[8–12]. They also impair the production of pro-inflammatory cytokines, chemokines and adhesion molecules[13–16]. These two actions allow the survival of the invading *Yersinia* and their extracellular multiplication in lymphoid tissues[17] (for a review see REF. 18). This review describes our current understanding of the Ysc INJECTISOME, the injection process and the intracellular action of the six known Yop effectors. The emphasis will be on the translocation of the effectors across the eukaryotic cell membrane and on the cell biology of the effectors.

Box 1 | Distribution of type III secretion systems among pathogenic bacteria



Type III secretion (TTS) systems have been discovered in many Gram-negative bacteria that are pathogenic for animals, plants and even insects[3–6]. Two systems are found in symbiotic bacteria (*Rhizobium* and *Sodalis glossinidius*). For *Sodalis*, these two systems are of the Inv Mxi–Spa type (see figure). Comparing the genes and their organization, TTS systems can be grouped in five 'families' (see figure). Interestingly, some bacteria harbour not only one system, but two from different families (*Salmonella enterica*, *Yersinia enterocolitica* from biogroup 1B, *Y. pestis* and *Burkholderia*)[102]. In *Salmonella*, the two systems come into play at different stages of the infection process. In the other cases, the role of the second system is not known. A recent phylogenetic analysis showed that the evolutionary tree of TTS systems differs completely from the phylogeny of their bacterial host, and hence that they have been distributed in bacterial populations by horizontal transfer[102]. In accordance with this hypothesis, they are generally found on plasmids (*Yersinia, Shigella*) or on discrete 'pathogenicity islands' in the bacterial chromosome.

The figure shows a phylogenetic tree of a TTS system (right) compared with the bacterial phylogenetic tree based on 16S RNA. The TTS system phylogenetic tree is based on sequence analysis of the cytoplasmic membrane-embedded proteins of the YscV family. TTS systems can be grouped into five families, excluding the TTS system of the flagellum. For each family, there is a schematic representation of the injectisome, some of which look very similar from electron-microscopy data. A simplified evolutionary tree of bacteria based on 16S RNA sequences is shown on the left. Animal/human pathogens are shown in red, and plant pathogens are in green. Insects pathogens or symbionts are in blue. Coloured arrows show the distribution of TTS systems of the different groups between the various pathogens. The many crossings of the arrows indicate that the two phylogenetic trees are completely unrelated[102].

INJECTISOME
The organelle responsible for 'secretion' of virulence proteins by the 'type III secretion' mechanism.

BASAL BODY
The basal body of the flagellum is the part that is embedded in the cell surface and that is in the bacterial cytoplasm. It consists of a rod, a set of four thin rings (L, P, S and M), and a large ring (C) that contains the flagellin-export apparatus.

PEPTIDOGLYCAN
Peptidoglycan is the rigid, shape-determining complex polymer that forms the cell wall of bacteria. It is made of chains of heteropolysaccharides linked by tetrapeptides.

FLAGELLUM
The locomotive organelle of bacteria. It consists of a basal body and a long hollow filament that is rotated by a molecular motor.

F_0F_1 PROTON TRANSLOCASE
A large and complex enzyme in the mitochondrial inner membrane that catalyses the synthesis of ATP, which is driven by a flow of protons.

Basic components of the Ysc–Yop TTS system

The Ysc–Yop TTS system consists of the Ysc (Yop secretion) apparatus — called the Ysc injectisome — and the array of Yops (*Yersinia* outer proteins) that are secreted by this apparatus. Three of the Yops — YopB, YopD and LcrV — translocate the effector Yops across the target-cell membrane[2,19] by forming some kind of pore[20,21]. So far, six Yop effectors have been identified: YopH, YopE, YopT, YpkA/YopO, YopP/YopJ and YopM. Four of them (YopH, YopE, YopT and YpkA/YopO) contribute to the impairment of phagocytosis by disturbing the dynamics of the cytoskeleton[11,22,23]; three of these (YopE, YopT and YpkA/YopO) act on monomeric GTPases of the Rho family[24–28], whereas YopH is a powerful protein tyrosine phosphatase[29] that dephosphorylates focal adhesions[10,30] and complexes that are involved in signalling adhesion[31]. In addition to its antiphagocytic role, YopH also inhibits lymphocyte proliferation[32] and the synthesis of monocyte chemotactic protein 1 (MCP1)[33]. YopP downregulates the inflammatory response of macrophages, epithelial and endothelial cells[14–16] by blocking the mitogen-activated protein kinase (MAPK)[14,15,34] and nuclear factor-κB (NF-κB)[15,35] pathways. Finally, the sixth effector — YopM — is still enigmatic. It is a protein comprised of leucine-rich repeats, and it has been shown to travel to the nucleus of the target cell[36]. Its role and target, however, are still unknown. Loss of any of the Yop effectors generally leads to a marked decrease in the virulence of *Yersinia* in infected mice.

A device to inject bacterial proteins into cells

The Ysc injectisome. Before contact with eukaryotic target cells, *Yersinia* incubated at the body temperature of their host build, at their surface, many copies of a secretion apparatus[37], which is known as the Ysc–Yop TTS system[38]. This secretion apparatus is an organelle that consists of a BASAL BODY, which spans the PEPTIDOGLYCAN layer and the two bacterial membranes, topped by a needle-like structure that protrudes outside the bacterium[39–43] (FIG. 1). The most internal part of the basal body includes the protein pump. By analogy with the FLAGELLUM (BOX 3), this part of the injectisome probably consists of a large cylinder and a membrane-spanning ring. YscN is an essential part of the pump[44]; it is an ATPase that resembles the β-catalytic subunit of F_0F_1 PROTON TRANSLOCASE. We can speculate that it polymerizes to form

Box 2 | **Infections caused by *Yersinia***

The genus *Yersinia* contains many species, but only three of them are pathogenic for rodents or humans — *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. *Y. pestis*, the agent of bubonic plague, has, in the past, caused social devastation on a scale unmatched by any other infectious agent (see figure). Although at present it is not an important public-health problem, plague has not disappeared and it is on the World Health Organization (WHO) list of re-emerging diseases. It is generally transmitted by a bite of the rat flea and then proliferates in the corresponding regional lymph node, which swells and becomes a 'bubo'. From there, it spreads through the lymph and blood, and overwhelms the patient with massive growth in the blood. At this stage, it might multiply in the lungs and become airborne transmitted, and it causes the death of healthy individuals within three days of infection.

Like *Y. pestis*, *Y. pseudotuberculosis* is also essentially a rodent pathogen. After oral inoculation, it causes diarrhoea, emaciation, and death as a result of septicaemia. *Y. enterocolitica* is a common human pathogen that causes gastrointestinal syndromes ranging from mild self-limited diarrhoea to mesenteric adenitis that resembles appendicitis. Contamination generally occurs by consumption of raw pork meat. Asymptomatic infections accompanied by mild bacteraemia are common. As *Y. enterocolitica* is a PSYCHROPHILIC bacterium, it might cause fatal blood-transfusion accidents.

In spite of the fact that they enter their host by different routes and cause diseases of different severity, all three pathogenic *Yersinia* have in common a tropism for lymphoid tissue and a remarkable ability to resist the primary immune response of the host. This capacity is essentially due to the Ysc–Yop type III secretion (TTS) system, which is described in the main text. Other virulence factors account for the differences in the severity of pathology between the three species, in particular the capacity to acquire iron[103,104]. The recent sequencing of the *Y. pestis* genome has shown the presence of a second TTS system[105], and a second TTS system has also been discovered in the most virulent biogroups of *Y. enterocolitica*[102,106]. Surprisingly, the second TTS systems found in *Y. pestis* and *Y. enterocolitica* are not the same. The role of these additional systems in virulence has not been addressed yet.

The figure shows images that relate to plague epidemics. Part a shows the costume that was worn by doctors during the plague epidemics in Marseille, France, in 1720. The epidemics killed half of the city's poplulation (~20,000 people). This costume was meant to protect the doctor, and the beak contained sweet-smelling herbs to 'filter' the contagious air. This figure is reproduced with the permission of The Granger Collection, New York. Part b shows a burial during the plague epidemics in Tournai, Belgium. It is an illustration from a manuscript dated 1348 (manuscript 13076–13077, folio 24, verso) © Brussels, Royal Library of Belgium.

the lower part of the cylinder, but this has not been shown. The distal part of the basal body is a ring-shaped structure, with a central pore of about 50 Å (REF. 45) that is formed by the polymerization of a protein known as YscC. The Ysc injectisome ends with a needle that is 600–800 Å long and 60–70 Å wide and is formed by the polymerization of a 6-kDa monomer called YscF. This needle has a hollow centre of about 20 Å (REF. 43). Mutants unable to synthesize YscF cannot secrete Yops[46], which indicates that the needle might keep the large YscC pore open. It is generally assumed that the injectisome serves as a hollow conduit through which the exported proteins travel to cross the two membranes and the peptidoglycan layer in one step. The injectisome as described here is sufficient for bacteria to release Yops into the environment, but it is not sufficient to translocate effector Yops across the eukaryotic cell membrane. Indeed, this step requires the translocator Yops (YopB, YopD and LcrV) (see below). It is, therefore, probably misleading to consider the injectisome as a syringe piercing the eukaryotic cell membrane on its own.

The injectisome can be artificially triggered to expel several Yops *in vitro* by chelating Ca^{2+} ions in the culture medium or by mutating some genes (*yopN*, *tyeA* and *lcrG*, see below). Yops are recognized specifically by the Ysc injectisome by a signal that is different from

PSYCHROPHILIC BACTERIA
Bacteria that can grow at 4 °C.

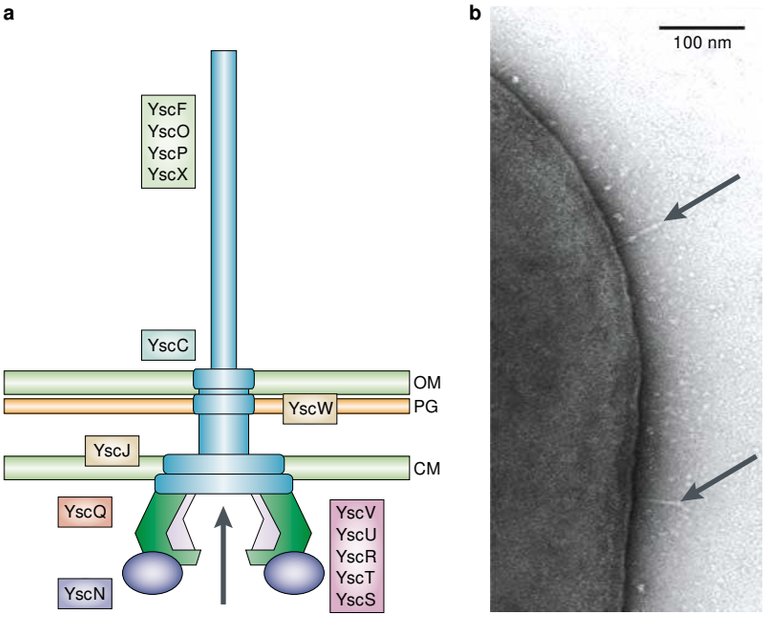


Figure 1 | **The Ysc injectisome. a** | Schematic representation of the Ysc injectisome spanning the outer membrane (OM), the peptidoglycan layer (PG) and the cytoplasmic membrane (CM) of the bacterium. The ring spanning the OM is made of the secretin YscC, assisted by the lipoprotein YscW. YscJ is another lipoprotein. YscF, YscO, YscP and YscX are external parts of the injectisome. YscF is the main constituent of the needle. YscV, YscU, YscR, YscT and YscS are proteins of the basal body that are in contact with the CM. YscN is the ATPase of the pump. YscQ is probably localized to the large inner cylinder. **b** | An electron micrograph of injectisome needles protruding from *Yersinia enterocolitica* E40. Image courtesy of L. Journet, P. Broz and G.R.C., University of Basel (Biozentrum), Switzerland.

the one that is recognized by the classical secretion pathway (the Sec pathway), although there is controversy about the nature of this signal (protein or messenger RNA) (BOX 4). Another peculiarity of the system is that several Yops need the assistance of specialized CHAPERONES to be secreted by the injectisome. Generally, these chaperones each serve only one Yop and they do not leave the bacterium (BOX 5).

Triggering the mechanism. *Yersinia* do not secrete Yops when they are incubated in a cell-free eukaryotic cell-culture medium. However, in the presence of target cells, they inject their Yop effectors, which indicates that physical contact with cells must be involved in triggering this process[1]. As the amount of intrabacterial Yops is strictly controlled by a feedback mechanism[47], as soon as they are released from the bacterium, synthesis resumes. This observation prompted the group of Wolf-Watz to place a luciferase gene under the control of the *yopE* promoter and to infect cells with the recombinant *Yersinia*[48]; it was observed that only adhering bacteria became luminescent, which elegantly showed the role of contact in triggering the system. Almost any type of animal cell can be a target; however, the injection of Yop effectors is strictly dependent on the capacity of the bacterium to adhere to the target[49].

For most cell types, adherence is achieved by means of specialized outer membrane proteins known as ADHESINS. Injection into specialized phagocytes, such as macrophages and PMNs, does not require the adhesins, presumably because the phagocytic receptors fulfil the adherence function[49]. This dependence on adhesins or receptors indicates that the system requires the docking of the bacterium on the target cell, and implies that this docking is not achieved by the needle itself. In the absence of docking, not only is there no injection of Yops, but the bacteria do not release Yops into the cell culture. Therefore, docking triggers secretion, but it is not known whether this stimulation involves a specific cell receptor. The involvement of such a receptor is, however, unlikely, as every cell type to which *Yersinia* can dock, by any kind of adhesion mechanism, receives an injection of Yops. Therefore, it seems that intimate contact with membrane lipids themselves might suffice to trigger secretion. On the bacterial side, however, three proteins called YopN[50], TyeA[22] and LcrG[51] seem to form two plugs that block the secretion channel at different levels. This concept is based on the observation that *yopN*, *tyeA* and *lcrG* mutants secrete Yops even in the absence of contact with a target cell.

Effector translocation across the eukaryotic cell membrane. Bacteria that are unable to synthesize the hydrophobic YopB and YopD proteins have no deleterious effects on target cells, because the Yop effectors are not translocated into the cytosol of these cells. So, YopB and YopD are required for the translocation of the effectors across the cell membrane. They are known as 'translocator' Yops to distinguish them from the effector Yops. When cultured macrophages are infected with bacteria that synthesize the translocators, but none of the effectors, they become permeable to small fluorescent dyes, flatten and then finally die by NECROSIS[21], which indicates that these translocators form pores.

On the basis of the size of the molecules that can leak in or out of such infected cells, the pores are thought to have a diameter of 16–23 Å (REFS 20, 21). Pores are not observed after infection with wild-type bacteria, which has led to the interpretation that the translocating effectors obstruct the pore[20,21]. Recently, Viboud and Bliska[52], working with HeLa cells, have shown that YopE, but not its 'catalytic' mutant, can mask the pore. As YopE depolymerizes actin (see below), it was proposed that translocated YopE inhibits actin polymerization to prevent membrane damage of infected cells[52]. An alternative explanation would be that pores can only be detected in cells where the membrane is stretched out by STRESS FIBRES. Using electrophysiology, pores have also been detected in artificial liposomes that have been incubated in the presence of *Y. enterocolitica* that is secreting YopB and YopD[53].

On the basis of all of these observations, it was concluded that the effectors travel across the eukaryotic cell membrane through a pore that is formed by the translocators. So far, however, this pore has not been observed by electron microscopy, and the stoichiometry and organizition of the protein components remain unknown. A third translocator Yop, LcrV, which is encoded by the same operon as YopB and YopD, but has

CHAPERONES
Several families of proteins, known as molecular chaperones, that assist nascent proteins in their folding or prevent premature or illicit associations with other proteins, folded or partially folded. Some chaperones, such as those involved in type III secretion, are small (~15 kDa) proteins without any ATP-binding site, whereas others, known as chaperonins, form large heteropolymeric cylinders and consume ATP.

ADHESINS
Bacterial proteins that promote adherence to host-cell membranes. Some are simply anchored in the bacterial membrane, whereas others are placed at the tips of pili.

NECROSIS
Death in response to cell or tissue damage, which ends in the release of the intracellular content and the onset of inflammation.

Box 3 | Does the injectisome derive from the flagellum?

About one third of the proteins that make up the injectisome have a homologue in the bacterial flagellum[107]. This bacterial locomotion apparatus consists of a long rotating filament — the flagellum itself — attached by a hook to a rotary motor that is anchored in the bacterial cell wall by four rings. The motor forms a 'bulb' inside the bacterium, and, in addition to the motor, this bulb contains the pump, which expels monomeric flagellins (the protein components of the flagellum). This pump is a type III secretion (TTS) apparatus, and it contains most of the flagellar proteins that have counterparts in the injectisome. Flagellin is secreted in a monomeric form through the flagellum itself and polymerizes at the tip (for review see REF. 108). So, the injectisome and the flagellum are two bacterial organelles that share both a TTS system and a few proteins that are involved in sorting the proteins to be secreted[107]. The recent electron-microscopy pictures of the complete *Salmonella*[42] and *Shigella*[40] injectisomes show that they include a basal body that is similar to that of the flagellum. The similarities between the basal body of the injectisome and the flagellum are marked and indicate a common origin. It is probably that the flagellum is the ancestor, given that the injectisome would be useful only after the appearance of unicellular eukaryotes.

Interestingly, the ring of the injectisome that spans the bacterial outer membrane is different from the equivalent ring of the flagellum basal body, and presumably has a different evolutionary origin. This ring structure has a large central pore of about 50 Å (REF. 45), made by the polymerization of a single protein known as YscC. YscC is related to a protein — known as PIV — that filamentous bacteriophages insert into the bacterial outer membrane to allow their extrusion from the bacterium[109].

no particular hydrophobicity, is also required for pore formation[54–58] (M. N. Marenne and G.R.C., unpublished observations). In addition to their role as translocators, YopD and LcrV both have regulatory effects on other components of the system, which makes the analysis of the pore by a genetic approach extremely complex[59,60].

The fact that the Ysc injectisome ends with a long needle that protrudes from the bacterium raises the question of the relationship between the needle and the pore-formers. As the injection process requires a very tight adhesin-mediated contact, the needle must either retract, break down or pierce the target-cell membrane. According to Hoiczyk and Blobel[43], the needle is sufficiently hydrophobic to pierce the membrane on its own. However, this conflicts with the absolute requirement of YopB and YopD for translocation[1,2,20,61]. To reconcile the various observations, one could hypothesize that YopB, YopD and LcrV destabilize the host-cell membrane, which then allows the needle to pierce it, driven by the pressure that gradually builds up as a result of increased docking of the bacterium at the cell surface. A few molecules of the translocators could already be present at the tip of the injectisome needle before contact, but this has not been shown. Alternatively, the pore-formers could be the first Yop proteins that are secreted after contact is established with a target cell.

Whether the needle penetrates the target-cell membrane or not, there are good arguments to support the idea that Yop effectors are guided through a continuous channel from the bacterial cytosol to the target-cell cytosol. Indeed, knockout bacteria lacking all of their effectors can deliver a reporter protein that contains only the first 15 residues of YopE into eukaryotic cells[62]. This shows that once a Yop has entered the Ysc secretion channel, it goes all the way to the eukaryotic cytosol — no specific signal or binding domain is required to cross the pore. However, this is not true for wild-type bacteria, in which YopE needs both its chaperone and chaperone-binding site to be delivered efficiently[63]. This indicates that the chaperone is needed when there is competition between several Yops for secretion and that it facilitates the entry of its partner Yop into the secretion channel[62] (BOX 4).

Mechanisms of Yop-protein action

Phagocyte paralysis by YopH, YopE, YopT and YpkA/YopO. As mentioned above, four effectors (YopH, YopE, YopT and YpkA/YopO) out of the six that have been identified so far, exert a negative effect on cytoskeletal dynamics, and in doing so contribute to the strong resistance of pathogenic *Yersinia* to phagocytosis by macrophages[8,9,11,64] and PMNs[12,64,65]. The actions of these effectors are summarized in FIG. 2. Videos of macrophages transfected with green fluorescent protein (GFP)–actin illustrate the cytoskeletal reorganization that is involved in phagocytosis, as well as the speed of the phenomenon (see Movies 1 and 2 online). A video of fibroblasts transfected with GFP–actin illustrates the total destruction of the cytoskeleton that occurs as a result of Yop action (see Movie 3 online).

YopH is one of the most powerful known phosphotyrosine phosphatases (PTPases)[66]. The carboxy-terminal domain (residues 206–408) resembles eukaryotic phosphatases, and it contains a phosphate-binding loop that includes a crucial cysteine residue (Cys403)[67]. Mutation of Cys403 converts YopH into an inactive enzyme that can still form complexes with substrates in infected cell cultures. The amino-terminal domain resembles other proteins of the Ysc–Yop system (for example, LcrQ/YscM1 and YscM2, which are not discussed in this review), and it contains the secretion signal (residues 1–17), as well as the domain (residues 17–71) that binds the YopH-specific chaperone SycH[2,68]. Surprisingly, phosphotyrosylpeptide-binding activity also localizes to this amino-terminal domain[69,70]. In this respect, YopH is reminiscent of the eukaryotic signalling proteins that contain separate PTPase PHOSPHOTYROSINE-BINDING (PTB) DOMAINS, but there is no detectable sequence homology, or even structural similarity[71,72], between the amino-terminal domain of YopH and eukaryotic PTB domains, such as SH2 DOMAINS. There is also no obvious cavity to accommodate phosphotyrosine residues in the vicinity of the residues that have been found to be important for binding tyrosine-phosphorylated $p130_{cas}$. When injected into J774 macrophages, YopH dephosphorylates the focal adhesion protein $p130_{cas}$ and disrupts focal adhesions[73] (FIG. 2). As deletion of residues 223–226 of YopH prevents it from being targeted to focal adhesions (at least in the less-relevant HeLa cells) and simultaneously impairs the antiphagocytic activity of *Yersinia* against macrophages[74], it is probable that the action of YopH against focal adhesions is relevant to its antiphagocytic action. Other targets have been identified in J774

STRESS FIBRES
Long axial bundles of actin microfilaments that run along the entire length of the cell.

PHOSPHOTYROSINE-BINDING (PTB) DOMAINS
Domains that bind phosphotyrosine residues and allow signalling interactions.

SH2 DOMAINS
Src-homology 2 domains are PTB domains that bind to phosphotyrosine residues, such as those that are found in activated receptor- or cytoplasmic-tyrosine kinases, and are involved in signalling processes.

Box 4 | The type III secretion signal controversy

As soon as it became clear that Yops are proteins that are secreted by a new secretion mechanism, now known as type III secretion (TTS), Michiels *et al.*[110] noticed that Yops are recognized through their amino terminus, and that no classical signal sequence is cleaved off during Yop secretion. The minimal region shown to be sufficient for secretion was reduced gradually to around 10–15 residues[63,111]. However, the idea that TTS signals reside in the secreted protein itself was challenged by Anderson and Schneewind[111,112], who carried out a systematic mutagenesis of the amino-terminal secretion signal of three Yops. These authors did not observe any point mutation that specifically abolished secretion, and they observed that even frameshift mutations failed to prevent secretion. They concluded from these observations that the secretion signal is encoded in the messenger RNA rather than in the peptide sequence.

This conclusion is at odds with two other observations. Indeed, bacteria incubated at 37 °C contain Yops[8], even in conditions in which they do not secrete them, which indicates that secretion has to be at least partially post-translational. The second problem comes from kinetics. Indeed, the antiphagocytic response has to occur within seconds after contact with a phagocyte, and it seems that injecting preformed Yops might be faster than synthesizing them on contact.

The mRNA-signal hypothesis was contradicted recently by Lloyd and collegues[113], who showed that mutations in the first 11 codons of *yopE* that modify the mRNA, but not the amino-acid sequence, do not impair secretion, indicating that it is indeed the amino-terminus of YopE, and not the 5′ end of *yopE* mRNA, that serves as a targeting signal. At the present time, therefore, it seems that the signal is in the protein rather than in the mRNA, and that secretion can be post-translational. However, the matter remains controversial[114].

macrophages. The Fyn-binding protein Fyb[73] and the scaffolding protein SKAP-HOM interact with each other and become tyrosine-phosphorylated in response to macrophage adhesion[31], and their dephosphorylation by YopH might allow *Yersinia* to interfere with an adhesion-regulated signal-transduction pathway in macrophages (FIG. 2). YopH also suppresses the oxidative burst in macrophages[8]. The effect of the Ysc–Yop system on PMNs has not been investigated as thoroughly as in macrophages; however, it clearly inhibits phagocytosis by PMNs [12,64], and YopH has been shown to reduce (contact-induced?) calcium signalling in these cells[65] (it is this signalling that leads to phagocytosis).

The three other Yop effectors that inhibit phagocytosis — YopE, YopT and YpkA/YopO — act on monomeric GTPases of the Rho family. These GTPases belong to a large group of eukaryotic GTPases that act as molecular switches and control key events in the cell by oscillating between their 'on' and 'off' forms according to the bound nucleotide. Replacement of the nucleotide is catalysed by various specialized proteins, including GTPase-activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs). Rho-family proteins are anchored to the inner side of the plasma membrane of the cell by a prenyl group that is covalently attached at the carboxy-terminal end of the protein. This attachment is essential for their function. Members of the Rho family that are known to control the dynamics of the cytoskeleton are Rho, Rac and Cdc42 (for a review, see REF. 75), and such monomeric GTPases have not been described in bacteria. YopE acts as a GAP, switching RhoA, Rac AND Cdc42 to their 'off' form by accelerating GTP hydrolysis[26,76] (FIG. 2). YopE has an arginine-finger motif similar to those found in eukaryotic GAP proteins, and exchanging Arg144 from this motif with an alanine residue results in the loss of both GAP activity and the ability to induce cytotoxicity in cultured cells[24,76]. The GAP activity of YopE is equally effective on RhoA, Rac and Cdc42 *in vitro*[76], but it is predominantly active on Rac *in vivo*, as shown in primary endothelial cells[24]. This GAP activity gives YopE its antiphagocytic function[26].

YopT has a strong depolymerizing effect on actin[22], as it modifies Rho-family proteins and displaces them from the membrane to the cytosol[27,77] (FIG. 2). It has been shown recently that YopT is a cysteine protease that cleaves RhoA, Rac and Cdc42 close to their carboxyl terminus, which releases them from their membrane anchor[28]. Therefore, YopT inactivates the Rho GTPases by detaching them from the membrane, rather than by blocking their cycling between GDP- and GTP-bound forms as YopE and several bacterial toxins do[78] (FIG. 2).

YpkA (*Yersinia* protein kinase A; known as YopO in *Y. enterocolitica*) modulates the dynamics of the cytoskeleton[23] (FIG. 2). It is an 80-kDa autophosphorylating serine/threonine kinase[79], which has some sequence homology and structural similarity to RhoA-binding kinases[80], and only becomes active by interacting with actin[81] or other eukaryotic factors that are present in HeLa-cell extracts and fetal calf serum[80]. In addition to being an activator of YpkA, actin can also function as an *in vitro* substrate of the kinase. YpkA/YopO interacts with RhoA and Rac1 irrespective of the nucleotide bound, and apparently does not affect their guanine nucleotide exchange capacity[80,25]. Although binding of YpkA/YopO to actin and to RhoA and Rac1 clearly seem to be relevant observations in the context of phagocytosis inhibition, both the kinase target and the exact mode of action of YpkA/YopO remain unknown.

Non-OPSONIZED *Yersinia* lacking just one of these four Yops are more readily phagocytosed by PMNs and by J774-cultured macrophages than are wild-type *Yersinia*, which indicates that all four of these Yops contribute to antiphagocytosis and that there is no redundancy between them — instead, there is synergism[64]. It is unclear which Yop is the most important. When *Yersinia* are opsonized with the complement fragment C3b or with immunoglobulin G, the removal of YopE seems to have little effect on phagocytosis by J774 macrophages, which indicates that YopE counteracts non-opsonic phagocytosis more efficiently than opsonic phagocytosis. In addition, the four different Yop mutants behaved in the same way whether they were opsonized by C3b or by IgG, indicating that no Yop was more efficient against one opsonic phagocytosis pathway than against the other[64].

Blocking the pro-inflammatory response of infected cells. Two intracellular effectors — YopP (YopJ in *Y. pseudotuberculosis* and *Y. pestis*) and YopH — have been shown to counteract the pro-inflammatory response of infected cells, and great attention has been paid recently to YopP/J. Although this effector has a remarkably strong effect on infected cells, evidence for its role in the

RhoA, Rac AND Cdc42
Monomeric GTPases that are involved in the control of actin polymerization/depolymerization.

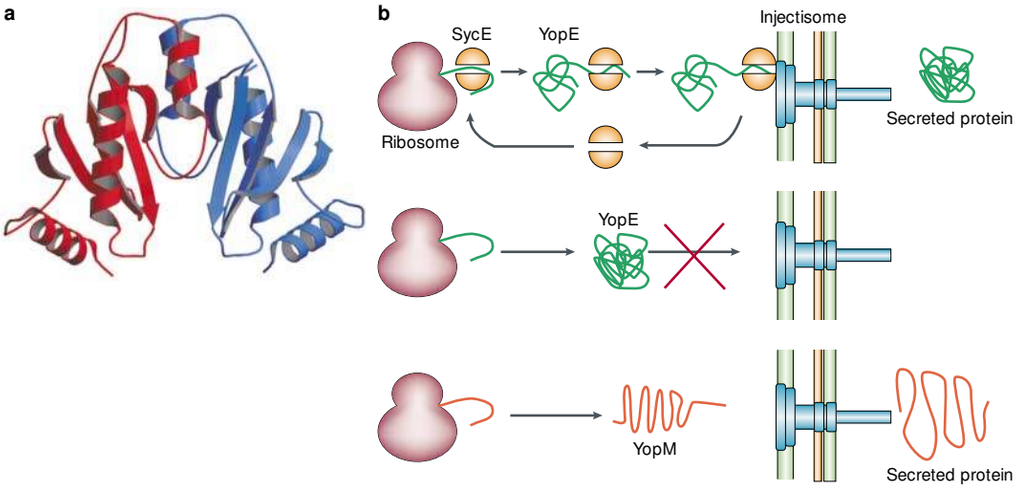
OPSONIZATION
The process by which IgG or complement C3b molecules bind to and coat particles, which enhances the efficiency of phagocytosis.

APOPTOSIS
Programmed cell death governed by complex signalling pathways and marked by a well-defined sequence of morphological changes, resulting in small bodies that are phagocytosed by other cells.

animal-infection model remains scarce. One report, however, showed that for *yopP/J*-mutant bacteria the dose of bacteria that is lethal to 50% of animals (LD_{50}) is 64-fold higher in mice than the LD_{50} for wild-type YopP/J, and that YopP/J causes APOPTOSIS of macrophage-antigen-1-positive cells from the mesenteric lymph nodes of infected mice[82]. However, in other reports, there was no difference in mouse lethality on infection with YopP/J-positive or -negative strains of *Yersinia*[16,83,84].

YopP/J counteracts the normal pro-inflammatory response of various cell types *in vitro* (FIG. 3). It reduces the release of tumour-necrosis factor (TNF) by macrophages[15], and of interleukin-8 (IL-8) by epithelial[35] and endothelial[16] cells. It also reduces the presentation of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1) and E-selectin at the surface of endothelial cells[16], and so presumably reduces the recruitment of PMNs to the site of infection. All of these events result from the inhibition of the activation of NF-κB — a transcription factor that is known to be central to the onset of inflammation[15,35,85]. The inhibition of NF-κB activation correlates with the inhibition by YopP/J of the inhibitor of

Box 5 | A new type of chaperone



The secretion of some Yops requires the presence of a new type of small cytosolic chaperone[68,115,116]. These are small acidic proteins with little or no sequence homology between them, but they have a putative carboxy-terminal amphiphilic α-helix. They also bear no sequence or functional resemblence to ATP-dependent chaperones, such as heat shock protein 70. They specifically bind only to one partner Yop, and in their absence, secretion of that Yop is severely reduced or abolished.

The chaperones in the *Yersinia* Ysc–Yop system are called the Syc proteins (specific Yop chaperone). Generally, they are encoded by a gene that is located next to the gene encoding the protein they serve. A lot of research effort has concentrated on YopE–SycE. SycE binds as a dimer to amino-acid residues 15–75 of YopE[63,115,117], and these residues are just downstream of the secretion signal. Some observations indicates that SycE could maintain pre-formed, stored YopE in a 'secretion-competent' state[113]. Indeed, the observed internal diameter of the needle is too small to allow folded, globular proteins to travel through it. If Yops are to travel through the needle, they have to be at least partially unfolded. Therefore, SycE might prevent YopE from folding prematurely.

In good agreement with this hypothesis, mouse dihydrofolate reductase (DHFR) — which is a cytosolic globular protein — can be secreted by the *Yersinia* Ysc injectisome as a YopE–DHFR hybrid protein only if SycE and the minimal SycE-binding domain are present[118]. However, this view is contradicted by the fact that binding of SycE to YopE does not abolish the Rho GTPase-activating protein (GAP) activity of YopE, which indicates that binding of SycE does not prevent YopE folding[117]. In addition, recent elucidation of the three-dimensional (3D) structure of SycE bound to the SycE-binding domain of YopE indicates that the influence of the chaperone does not extend to the GAP domain of YopE[117]. These results indicate that the YopE–SycE complex could form some kind of secondary 3D secretion signal that helps secretion that is initiated by the amino-terminal signal[117]. This idea is consistent with the view that SycE could be some kind of a hierarchy factor[62].

So, in spite of many elegant experiments and important structural achievements, there is still no clear answer to the question of the role of SycE. The situation is no different for the other Syc, some of which might not necessarily have the same role as SycE does for YopE. However, one intriguing question remains unanswered regarding all of these chaperones — which protein of the injectisome strips the Yops of their partner chaperone as they enter the channel?

The figure shows the 3D structure of the SycE chaperone (part a) and a model for the role of SycE (part b). SycE binds to the amino terminus of nascent YopE and allows YopE to be secreted by the injectisome (part b, upper panel). In the absence of SycE, YopE either adopts a conformation that is incompatible with secretion or is not properly presented to the injectisome and is degraded (part b, middle panel). In contrast to YopE, YopM does not need a chaperone to be secreted by the injectisome (part b, lower panel). Part a is reproduced with permission from REF. 119 © (2002) Macmillan Magazines Ltd.

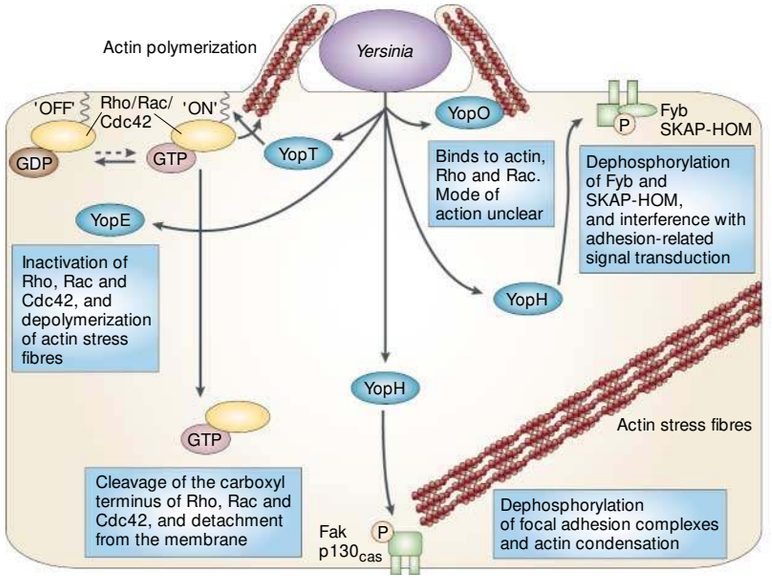


Figure 2 | **Targets of YopE, YopH, YopT and YpkA/YopO.** YopH is a powerful protein tyrosine phosphatase that dephosphorylates Fak and $p130_{cas}$ in focal adhesion complexes, as well as Fyb and SKAP-HOM in an adhesion-signalling complex. YopE exerts a GTPase-activating protein (GAP) activity on monomeric GTPases from the Rho family (Rho, Rac and Cdc42), and YopT cleaves the same monomeric GTPases close to their carboxyl terminus, which releases them from their membrane anchor. The actions of YopE and YopT both result in the inactivation of Rho proteins and the depolymerization of actin stress fibres. YopO is a kinase with homology to eukaryotic serine/threonine kinases that is activated by actin binding and binds RhoA and Rac1. Its mode of action is unclear at present.

NF-κB kinase β (IKKβ) — a kinase that phosphorylates the inhibitor of NF-κB (IκB)[86] (FIG. 3). By preventing the phosphorylation of IκB, YopP/J prevents the degradation of IκB and so prevents the translocation of NF-κB to the nucleus (FIG. 3).

In addition to acting on the NF-κB pathway, YopP/J also inhibits the activation of the MAPKs c-jun-N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase 1 (ERK1) and ERK2[14,15,34]. This lack of activation of the MAPKs results from the inhibition of the upstream MAPK kinases (MEKs)[86] (FIG. 3). Inhibition of the MAPK pathways abrogates phosphorylation of the cAMP-response-element-binding protein (CREB), another transcription factor that is involved in the immune response[87] (FIG. 3). Not surprisingly, in view of its effect on the MAPK and NF-κB pathways, YopP has been shown recently to modulate the expression of at least 37 macrophage genes[88], most of which are known to be involved in the immune response.

YopP/J induces apoptosis in macrophages, but not in other cell types[89,90]. This apoptosis involves the classical cascade of signalling events that are observed when apoptosis is triggered by an extracellular signal. It is accompanied by cleavage of the cytosolic protein Bid — a pro-apoptotic member of the Bcl-2 family of proteins — which triggers the release of cytochrome *c* from mitochondria. This, in turn, leads to the cleavage of caspase-9, -3 and -7, which executes cell death[91] (FIG. 3). It is unclear so far whether apoptosis results from a YopP/J-induced early-cell-death signal or from the YopP/J-induced loss of NF-κB activity, which is known to protect cells from apoptosis[92] (FIG. 3).

Using the observation that the predicted secondary structure of YopP/J resembles that of an adenovirus protease, studies[93] were carried out that managed to identify and mutate crucial cysteine and histidine residues in the putative protease catalytic site. Mutations in this site prevented the anti-inflammatory and pro-apoptotic activities of YopP/J described above[91,93], which indicates that YopP could be a protease. YopP/J also has limited sequence homology to the yeast ubiquitin-like protease Ulp1, which cleaves the carboxyl terminus of an 11-kDa small ubiquitin-related modifier (SUMO1)[93]. SUMO1 is the best-characterized member of a rapidly growing family of ubiquitin-like proteins that are involved in post-translational modification. To verify whether YopP/J could act as a SUMO-protease, Orth *et al.*[93] simultaneously transfected cells with YopP/J and SUMO1, and observed that YopP/J reduced the cellular concentration of SUMO1-conjugated proteins and also reduced the amount of free SUMO1.

Although this indicates that YopP/J could be a SUMO protease, this behaviour is not typical of SUMO proteases. In addition, the proteins that YopP/J has been shown to bind are not known to be SUMOylated. So, how could the SUMO-protease hypothesis be reconciled with the previous findings? Orth *et al.*[93] have proposed that MEKs or IKKβ could escort YopP/J to signalling complexes in the cell, where it could exert its protease activity on an unknown SUMOylated substrate, which would, in turn, prevent the phosphorylation of MEKs and IKKβ. Obviously, the final solution to this problem awaits the identification of the proteolytically cleaved substrate(s).

YopP/J is not the only Yop that downregulates the inflammatory response. Recently, it has been shown that YopH prevents the synthesis of the MCP1 by infected macrophages[33] (FIG. 3). This inhibition could have a crucial role during infection by inhibiting the recruitment of monocyte-derived macrophages to lymph nodes[94]. Inhibition of MCP1 synthesis results from inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by YopH, through an as-yet-unknown mechanism[33] (FIG. 3).

Inhibition of lymphocyte proliferation. Yao and colleagues[32] observed that T and B cells that are transiently exposed to *Y. pseudotuberculosis* are impaired in their ability to be activated through their antigen receptors. In response to antigenic stimulation, T cells are inhibited in their ability to produce cytokines, and B cells are unable to upregulate surface expression of the co-stimulatory molecule B7.2 (CD86). This block of activation results from the inhibition of early phosphorylation events[32]. Through the analysis of various mutants, YopH seems to be the main effector involved in these events[32], and the recent observation that YopH inhibits the PI3K pathway[33] might account for this, as the PI3K pathway is known to control cell proliferation. So, YopH not only contributes to evasion of the innate immune response by inhibiting phagocytosis,

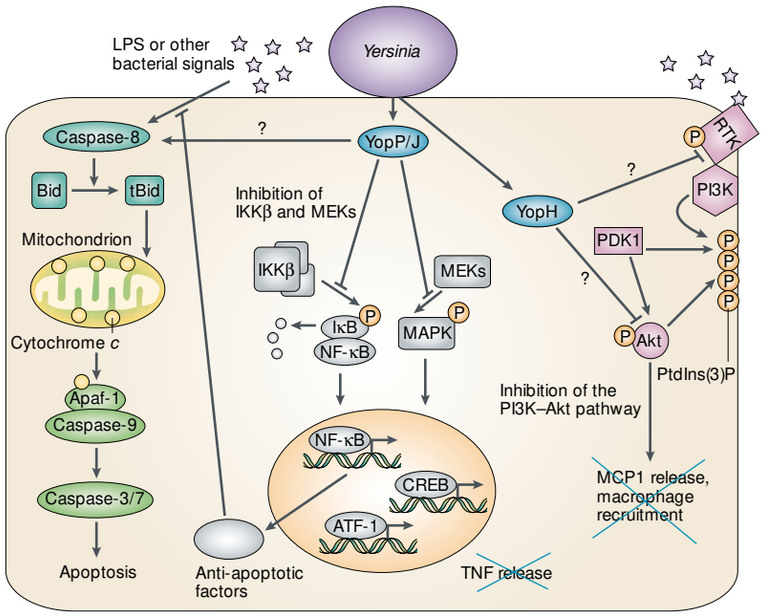


Figure 3 | **The anti-inflammatory action of Yops.** On infection of macrophages with *Yersinia enterocolitica*, Bid — a pro-apoptotic member of the Bcl-2 family of proteins — is cleaved to its pro-apoptotic truncated form (tBid), presumably by activated caspase-8. The protein tBid then triggers the release of cytochrome *c* from mitochondria, which activates the apoptotic protease activating factor-1 (Apaf-1), which activates caspase-9. Caspase-3 and -7 are then activated in turn, which promotes apoptosis. YopP/J is thought to activate apoptosis through one of two routes — either by preventing the release of anti-apoptotic factors through its anti-inflammatory effects (see below) or through a more 'direct' pathway. YopP/J inhibits the migration of nuclear factor (NF)-κB to the nucleus by preventing the phosphorylation of IκB, the inhibitor of NF-κB. This inhibition of phosphorylation results from the inhibition of the kinase of IκB (IKKβ). YopP/J also inhibits the mitogen-activated protein kinase (MAPK) pathways by inhibiting the upstream MAPK kinases (MEKs). As a result of these inhibitory actions, transcription activators such as the cAMP-response-element-binding protein (CREB) and activating transcription factor (ATF)-1, as well as NF-κB, cannot stimulate the transcription of genes that are involved in the synthesis of pro-inflammatory cytokines and adhesion molecules. The link between the pro-apoptotic role (left) and the anti-inflammatory role (middle) of YopP/J remains mysterious, as does the exact mode of action of YopP/J. YopP/J seems to be a protease, possibly of the de-SUMOylating family (where SUMO stands for 'small ubiquitin-related modifier'), but how the protease activity relates to the inhibition of the NF-κB and MAPK pathways is not known. The right hand part of the figure shows that, on contact of lipopolysaccharide (LPS) or other bacterial signals with an unidentified receptor tyrosine kinase (RTK), phosphatidylinositol 3-kinase (PI3K) is recruited to the membrane. Activated PI3K phosphorylates lipid phosphoinositides, leading to the appearance in the membrane of phosphatidylinositol-3-phosphates (PtdIns(3)Ps). These, in turn, recruit proteins such as phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates and activates Akt (also known as protein kinase B). Activated Akt, in turn, can phosphorylate different classes of proteins, which leads, among many other events, to the synthesis of monocyte chemotactic protein 1 (MCP1). TNF, tumour necrosis factor.

but it can also incapacitate the adaptive immune response of the host.

The enigmatic YopM. YopM is a strongly acidic protein containing 13–20 repeats of a 19-residue leucine-rich-repeated motif (LRR). Similar to other LRR-containing proteins, YopM has a crescent shape, formed from parallel β-sheets, with a loose amino terminus[95]. Intriguingly, four YopM monomers form a hollow cylinder with an inner diameter of 35 Å (REF. 95). YopM is an important virulence factor in *Yersinia* infection in mice[96,97], and it has been shown to be injected into target cells[61], where it migrates to the nucleus by means of a vesicle-associated pathway[36]. Its role and target are not known yet. However, a recent microarray analysis of infected macrophages showed that YopM reduces the transcription of several genes that are related to the cell cycle and cell growth, including the gene encoding B-myb, a transcription factor that stimulates the proliferation of haematopoietic cells[88].

LcrV — the red herring. Since the mid-1950s, LcrV has been known to be a protective antigen against plague[98]. Later on, it was found to be a Yop protein, and its gene is located with the genes for the translocators YopB and YopD. Recent work from several laboratories concluded that it interacts with YopB and YopD to promote the translocation of the effectors[54,57–59]. However, in addition to this crucial role in the delivery of the Yops, it has a regulatory role inside the bacterium[99]. To add to the complexity, it might also have a role on its own.

Indeed, intravenous injection of a fusion protein containing the 259 carboxy-terminal residues of LcrV into mice that had been previously induced by injection of non-virulent bacteria significantly reduced the levels of TNF and interferon-γ[100]. This immunosuppressive effect was confirmed recently by the observation that LcrV can suppress TNF in yeast-stimulated mouse or human macrophages[101], and the underlying mechanism was assigned to LcrV-mediated IL-10 production. The observation that IL-10-deficient mice are more resistant to *Yersinia* infection than are wild-type mice indicates that LcrV-mediated immunosuppression is relevant to the pathogenicity of *Yersinia*[101].

Unfortunately, the anti-inflammatory role of LcrV during mouse infection cannot be studied by the analysis of *lcrV*-knockout *Yersinia*, because of the role of LcrV in the regulation and translocation of the other Yops. Indeed, *lcrV*-mutant *Yersinia* do not inject any Yop proteins and so have completely lost their virulence. We are, therefore, facing the paradoxical situation that the translocated Yop effector YopP strongly inhibits the NF-κB and MAPK pathways, but seems to have a minor anti-inflammatory role *in vivo*, whereas LcrV, a component of the translocation apparatus itself, seems to have an important anti-inflammatory role *in vivo* .

Conclusion

Pathogenic *Yersinia* use a complex organelle to inject six effectors into the cells of the host that are involved in the innate immune response. As a result of this 'anaesthetizing' injection, phagocytosis is inhibited, recruitment of PMNs and monocyte-derived macrophages is reduced, and lymphocyte proliferation is prevented. No less than four effectors (YopH, YopE, YopT and YpkA/YopO) contribute to the antiphagocytic action, and there is no redundancy — rather, there is synergy[64] — which reflects the need for a fast and highly-efficient response to contact with a phagocyte. Three Yops (YopP/J, YopH and LcrV) contribute to the neutralization of inflammation and block the immune response. YopP/J inhibits the

MAPK and NF-κB pathways, thereby blocking the release of cytokines and the presentation of adhesion molecules. In addition to its antiphagocytic role, YopH has other important roles during infection, such as inhibiting the proliferation of lymphocytes and, perhaps, the recruitment of monocytes.

So, the actions of several of the Yops converge to single key issues, although some Yops have different effects. Understanding the role of YopM is still a challenge, as is understanding both the 'unplugging' of the system on contact with a target cell and the exact role of the translocator Yops and the needle in the process of Yop-effector translocation across the target plasma membrane. These questions belong to both microbiology and to cell biology, or, rather, to the new and blooming discipline of cellular microbiology.

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