

Review

Exploitation of Eukaryotic Ubiquitin Signaling Pathways by Effectors Translocated by Bacterial Type III and Type IV Secretion Systems

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ABSTRACT

The specific and covalent addition of ubiquitin to proteins, known as ubiquitination, is a eukaryotic-specific modification central to many cellular processes, such as cell cycle progression, transcriptional regulation, and hormone signaling. Polyubiquitination is a signal for the 26S proteasome to destroy earmarked proteins, but depending on the polyubiquitin chain topology, it can also result in new protein properties. Both ubiquitin-orchestrated protein degradation and modification have also been shown to be essential for the host's immune response to pathogens. Many animal and plant pathogenic bacteria utilize type III and/or type IV secretion systems to inject effector proteins into host cells, where they subvert host signaling cascades as part of their infection strategy. Recent progress in the determination of effector function has taught us that playing with the host's ubiquitination system seems a general tactic among bacteria. Here, we discuss how bacteria exploit this system to control the timing of their effectors' action by programming them for degradation, to block specific intermediates in mammalian or plant innate immunity, or to target host proteins for degradation by mimicking specific ubiquitin/proteasome system components. In addition to analyzing the effectors that have been described in the literature, we screened publicly available bacterial genomes for mimicry of ubiquitin proteasome system subunits and detected several new putative effectors. Our understanding of the intimate interplay between pathogens and their host's ubiquitin proteasome system is just beginning. This exciting research field will aid in better understanding this interplay, and may also provide new insights into eukaryotic ubiquitination processes.

Introduction

Ubiquitination is a fundamental post-translational protein modification for all eukaryotic organisms. It controls several critical aspects of cell metabolism, such as cell cycle progression, transcriptional regulation, signal transduction or recognition, and resistance to pathogens [1–3]. Ubiquitination consists of the conjugation of one or several ubiquitin (Ub) moieties onto a target protein (Figure 1). Monoubiquitination can trigger an alteration of the localization and/or the activity of a target protein [4,5]. Polyubiquitination can modulate the properties of the target protein or constitute a signal for its subsequent degradation

by the 26S proteasome [6]. As a general rule, the chains comprised of Ub moieties covalently linked together via their lysine residue (K) 48 are earmarked for proteasome-dependent degradation, whereas K63-linked chains are known to activate and modify protein activity and trafficking [4,5]. Cellular proteins can also be modified by a covalent link to Ub-like proteins (e.g., a small ubiquitin-related modifier [SUMO]; NEDD8). These Ub-like modifiers do not form multimeric chains and have been described to modulate protein properties [1,2]. The ubiquitination process involves successive enzymatic activities [4,5,7]: The Ub-activating enzyme (or “E1”) binds to the C-terminus of Ub in an ATP-dependent reaction via a cysteine residue in its active site. The thioester-linked Ub is then transferred to a cysteine residue of the Ub-conjugating enzyme (or “E2”). Different E2 Ub-conjugating enzymes seem to be responsible for the different (K48 and K63) poly-Ub chain topologies [7,8]. Eventually, the Ub ligase enzyme (or “E3”) controls the specificity of substrate ubiquitination by recruiting the target protein. E3 Ub ligases constitute a large protein family

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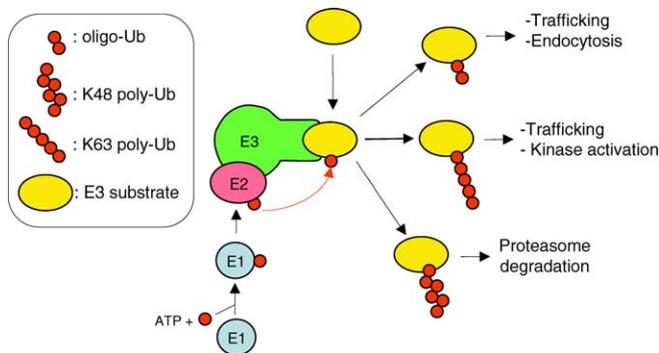
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Abbreviations: ARF, adenosine diphosphate ribosylation factor; ASK, *Arabidopsis* SKP1-like protein; FBP, F-box protein; GEF, GDP-GTP exchange factor; GTP, guanosine 5'-triphosphate; HECT, homologous to E6-AP C-terminus; HR, hypersensitive response; IκBα, inhibitor of NF-κB; IKK, inhibitor of NF-κB kinase; IL, interleukin; K, lysine residue; LRR, leucine rich repeat; MAMP, microbial-associated molecular pattern; MAPK, mitogen-activated protein kinase; MEKK, mitogen-activated protein kinase kinase kinase; NEMO, NF-κB essential modifier, or IKKγ subunit; NF-κB, nuclear factor κB; RING, really interesting new gene; SCF, Skp1/Cullin1/F-box, or E3 Ub ligase; SPI, *Salmonella* pathogenicity island; *S. typhimurium*, *Salmonella enterica* serovar Typhimurium; SUMO, small ubiquitin modifier; T3SS, type III secretion system; T3/4SS, type III and/or type IV secretion system; T4SS, type IV secretion system; TAB, TAK1-binding protein; TAK1, TGF-β-activated kinase 1; T-DNA, transferred DNA; TLR, toll-like receptor; TNFR, tumor necrosis factor receptor; TNFα, tumor necrosis factor α; TRAF, tumor necrosis factor receptor-associated factor; Ub, ubiquitin; UPS, ubiquitin proteasome system; VIP1, VirE2-interacting protein 1

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Figure 1. The Eukaryotic UPS

Schematic representation of signaling in the UPS, which requires a series of enzymatic steps involving E1, E2, and E3 enzyme complexes that will eventually lead to the addition of Ub moieties to target proteins. The specific recognition of substrates (yellow) by an E3 Ub ligase generally depends on the prior phosphorylation of the substrate (not indicated in the figure). Different types of ubiquitination can lead to different modifications, from proteasome degradation (for the K48-linked poly-Ub chain) to modification in protein properties (K63, oligo Ub [5]). Note that the scheme presented here applies to RING-type E3 ligases; in HECT-type E3s the Ub moiety is transferred from the E2 onto the conserved cysteine of the HECT protein, which then transfers this Ub onto the target protein (see text for details).

present in all eukaryotes and are distributed in two main groups, really interesting new gene (RING)-type, and homologous to E6-AP C-terminus (HECT)-type E3 Ub ligases. RING-type ligases directly and covalently attach the C-terminus of the Ub from an E2 to a lysine residue of the target protein, whereas the HECT-type protein forms a thioester bond with ubiquitin by its active cysteine residue before transferring it to a substrate. RING-type E3 Ub ligases can be either single (U-box type) or multi-subunit enzymes (generally cullin-based) [9]. When a substrate protein is K48-polyubiquitinated (with at least four subunits [10]), it is targeted to the cell proteasome, which unfolds the protein and degrades it into three to 20 residue peptides, which can be further degraded by downstream aminopeptidases [3,11–13]. Although archaeobacteria contain subunits homologous to the 20S proteasome [13], and bacteria contain likely ancestors of the E1 and E2 enzymes [14], the ubiquitination process as such is not found in prokaryotes.

Many Gram-negative pathogenic bacteria of both animals and plants have evolved type III and/or type IV secretion systems (T3/4SSs) as an essential virulence determinant. These secretion systems are large protein complexes spanning the bacterial envelope that are dedicated to the transfer of protein or DNA substrates into target cells to subvert host defense and other signaling cascades for the benefit of the invading pathogen. We refer the reader to some excellent reviews on these topics [15–17]. In the last decade, an enormous body of work has established the role of T3/4SS effector proteins in bacterial virulence. Similarity in structure or function to eukaryotic proteins allows them to interfere with many different cellular processes, including cytoskeleton rearrangement and intracellular trafficking. The biochemical functions they fulfill within the host cell remain undetermined for the plethora of bacterial T3/4SS effectors identified to date. This is a major challenge for understanding the molecular basis of pathogenicity. Recent advances in this

field include the discovery of the different T3/4SS effectors of mammalian bacterial pathogens that have the capacity to interfere with the host's Rho GTPase activity, to reorganize the actin cytoskeleton, and to allow or prevent bacterial internalization ([18] and references therein). Another example of the intriguing co-evolution between a pathogen and its host is the type III secretion system (T3SS) effector-mediated suppression of localized programmed cell death, which is triggered in plants when a specific resistance protein recognizes a specific avirulence protein of the pathogen ([19] and references therein).

In this review we focus on the growing number of T3/4SS effectors from both intracellular and extracellular plant and animal bacterial pathogens that specifically exploit their host ubiquitin proteasome system (UPS) (Table 1 and Figure 2). We want to illustrate the different mechanisms that these diverse bacteria have adopted to interfere with this key signaling component of the eukaryotic cell for the benefit of their specific infection strategy. First, we discuss the effectors that utilize the host UPS to ensure their own degradation or modification by ubiquitination, providing a means to regulate their concentration, and for the timing of their action. Second, we present effectors that share the ability to interfere with the ubiquitination level of key cell proteins of mammalian innate immune signaling cascades and, as a result, block the immune response. Third, we discuss two effectors of a plant pathogen that suppress plant innate immune responses, possibly by earmarking host proteins for degradation by the host UPS. Finally, we illustrate the subtle interplay between a pathogen and its host by mimicry of E3 ligase subunits, thereby manipulating the host UPS to the advantage of the pathogen.

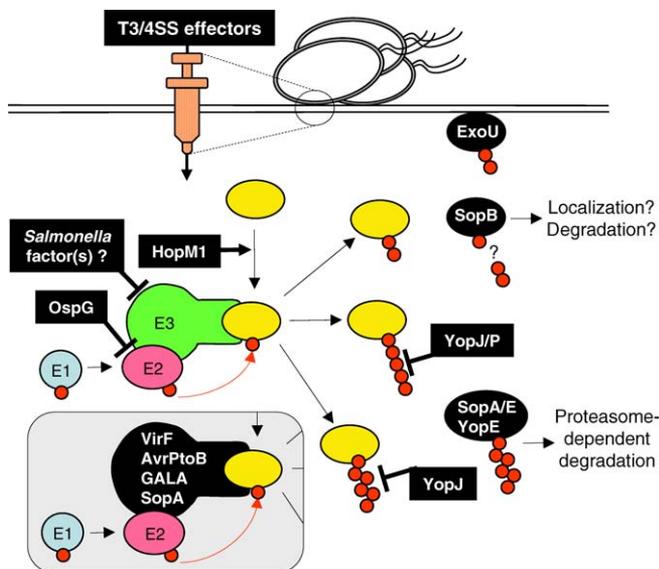
Bacterial Effectors Modulated or Degraded via Ubiquitination by the Host UPS

Recent reports suggest that exploitation of the host UPS could be a general mechanism used by bacteria to program the destruction of a T3/4SS effector when its function in the host cell is no longer required. This could be to temporarily activate a specific host protein or process, or to prevent deleterious effects to the host cell, which needs to stay in an optimal condition for bacterial colonization. The first lines of evidence for such a mechanism come from work on *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) T3SS effectors. This facultative intracellular bacterium is an important enteric pathogen of humans, causing gastrointestinal inflammation. Two T3SSs, encoded by the *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2), are essential for its pathogenicity and are used during different stages of infection for entry into intestinal cells and subsequent replication of the intracellular bacteria, respectively [20,21]. Among the range of translocated T3SS effectors encoded by SPI-1 are two proteins that alter the structure and the function of the actin cytoskeleton but exhibit opposing activities: SopE and SptP. SopE acts as a GTP-GDP (guanosine 5'-triphosphate-guanosine 5'-diphosphate) exchange factor (GEF) that activates the signaling molecules Rac-1 and Cdc42, two proteins of the Rho GTPase family, thus provoking cytoskeleton reorganization, which results in bacterial internalization. In contrast, the SptP effector functions as a GTPase-activating protein that deactivates Rac

Table 1. Bacterial T3/4SS Effectors Interfering with Their Host's UPS

Bacterial Protein	Bacterium	Host Protein	Ubiquitination	Proteasome Degradation	Mode of Action of the Effector	Role in Virulence	Reference
AvrPtOB	<i>Pseudomonas syringae</i> pv tomato	?	Autoubiquitination of AvrPtOB.	?	RING U-box E3-Ub ligase	AvrPtOB E3 Ub ligase required for virulence and cell death suppression.	[79,81]
ExoU	<i>Pseudomonas aeruginosa</i>	?	ExoU K63 bi-Ub	No	?	?	[35]
GALA	<i>Ralstonia solanacearum</i>	?	?	?	Plant-like F-box proteins	GALAs required for full virulence on different host plants.	[113]
HopM1	<i>Pseudomonas syringae</i> pv tomato	AtMIN7	AtMIN7	AtMIN7	Recruitment of AtMIN7 by HopM1 for degradation.	HopM1 suppresses host innate immunity.	[78]
OspG	<i>Shigella flexneri</i>	Ubiquitinated UbcH5b	Prevents phospho-IκBα K48-Poly-Ub.	Prevents phospho-IκBα degradation.	Inhibits SCF ^{trc} , binds UbcH5 E2.	OspG prevents phospho-IκBα ubiquitination and degradation.	[60]
SopA	<i>Salmonella typhimurium</i>	HsRMA1	SopA K48-poly-Ub	SopA	?	Ubiquitination of SopA by HsRMA1 is necessary for <i>Salmonella</i> escape from vacuoles.	[26]
SopA	<i>Salmonella typhimurium</i>	?	Autoubiquitination of SopA (mono-Ub)	?	SopA is a HECT E3 Ub ligase.	Mono-ub of SopA is not required for <i>Salmonella</i> escape from vacuoles, but is involved in transepithelial migration of neutrophils.	[27]
SopB	<i>Salmonella typhimurium</i>	?	SopB K48-poly-Ub	Proteasome-independent SopB degradation.	?	SopB induces actin polymerisation and bacterial entry.	[25]
SopE	<i>Salmonella typhimurium</i>	?	SopE K48-poly-Ub	SopE	?	SopE induces membrane ruffling, actin rearrangement, and bacterial internalization.	[23]
VirF	<i>Agrobacterium tumefaciens</i>	VIP1	?	Direct VIP1 and indirect VirE2 degradation by the proteasome.	F-box protein	Chemical inhibition of the proteasome, inhibits natural T-DNA transformation.	[98,100]
YopE	<i>Yersinia enterocolitica</i>	IKKβ	YopE K48-poly-Ub	YopE	?	?	[31]
YopJ	<i>Yersinia pseudotuberculosis</i>	IKKβ	IKKβ mono-Ub	No	Deubiquitinase	YopJ inhibits IKK complex, hence, inhibits NF-κB activation.	[45]
YopJ	<i>Yersinia pseudotuberculosis</i>	TRAF2, TRAF6, IκBα	TRAF2 and TRAF6 K63-poly-Ub and IκBα K48-poly-Ub.	Prevents degradation of IκBα.	Deubiquitinase	YopJ blocks inflammatory response by inhibiting NF-κB, and MAPK pathways.	[50]
YopP	<i>Yersinia enterocolitica</i>	TRAF6, NEMO	TRAF6, NEMO and TRAF6 K63-poly-Ub.	Prevents degradation of IκBα.	Deubiquitinase	YopP blocks inflammatory response by inhibiting NF-κB, and MAPK pathways.	[44]
YopP	<i>Yersinia enterocolitica</i>	TAK1, TAB1	TAK1, TAB1 ?	?	Binds TAB1 to inhibit TAK1 activity. Affects TAB1 and TAK1 ubiquitination.	YopP blocks inflammatory response by inhibiting NF-κB, and MAPK pathways.	[43]

All effectors listed are T3SS effectors except VirF (T4SS). K48 and K63 refer to the Ub lysine residue that is linked to the next Ub. A question mark means unknown. doi:10.1371/journal.ppat.0030003.t001



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Figure 2. T3/4SS Effectors Interfering with the Host UPS

After T3/4SS-mediated translocation into the host cell, several effectors (black-boxes) of diverse bacteria can target different steps of the UPS: some effectors have been shown to inhibit specific steps (e.g., inhibit an E3 Ub ligase, such as OspG, or deubiquitinate substrates like YopJ and YopP), mimic different UPS E3 Ub ligase subunits (here represented as one black subunit for simplicity as VirF, AvrPtoB, GALA, and SopA), or act as possible adaptor to target substrates to the E3 ligase (HopM1). Eventually, effectors can be a substrate for the UPS, like ExoU, SopB, SopE, and YopE (black ellipses). Table 1 describes in more detail the effectors presented in this figure. “*Salmonella* factor(s)?” refers to unknown factors from non-pathogenic and attenuated *Salmonella* strains (see text).

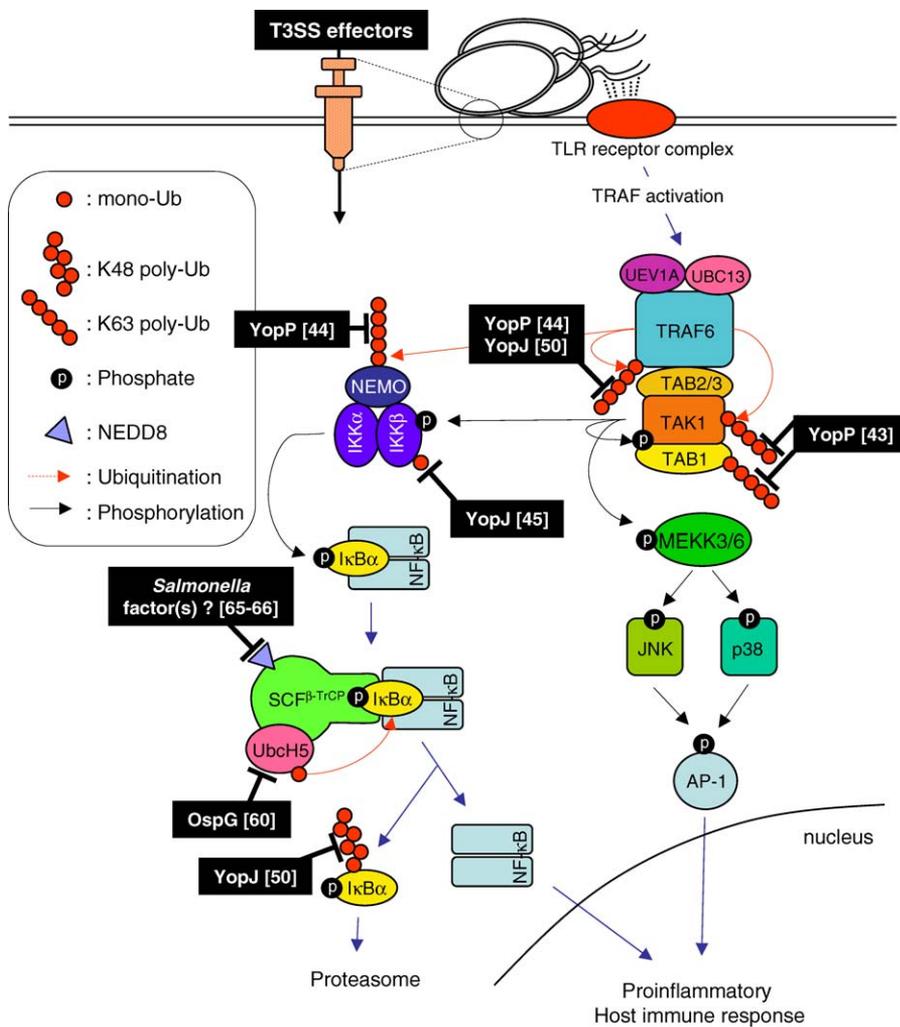
and Cdc42 [22], allowing the recovery of the actin cytoskeleton’s normal appearance a few hours after infection. For successful colonization, the activity of these two T3SS effector proteins has to be temporally regulated within the host cell. The mechanism of this regulation was shown to be due to their differential degradation by the host proteasome. SopE and SptP are delivered in equal amounts during infection, but SopE undergoes polyubiquitination and rapid proteasome-dependent degradation following translocation, whereas SptP is degraded at a much slower rate [23,24].

Two other *Salmonella* SPI-1 T3SS effectors, SopA and SopB, are functionally regulated by host ubiquitination. SopB is a phosphoinositide phosphatase that modulates vesicle trafficking by altering the phosphoinositide metabolism. It was shown to be monoubiquitinated and degraded, although probably not via the proteasome [25]. SopA, a protein required for the elicitation of intestinal inflammation, has been shown to be ubiquitinated within the host cell by the membrane-anchored RING-type E3 Ub ligase HsRMA1, and degraded by the proteasome in an HsRMA1-dependent manner [26]. The authors suggest that HsRMA1-dependent ubiquitination of SopA is involved in the escape of bacteria from *Salmonella*-containing vacuoles into the cytosol of epithelial cells. Recently, the same research group identified SopA as a HECT-type E3 Ub ligase. They identified the catalytic cysteine residue of SopA, and showed the formation of a transient E3-ubiquitin intermediate. These new data are in line with the mono-Ub state of SopA previously detected in the absence of HsRMA1 [26]. Interestingly, this SopA HECT

ligase activity is not required for the escape of bacteria from *Salmonella*-containing vacuoles into the cytoplasm, but seems to be involved in *Salmonella*-induced transepithelial migration of polymorphonuclear neutrophils (PMNs) [27]. The recruitment of these inflammatory cells depends on several factors, including interleukin (IL)-8 secretion, and is considered an important factor for the development of *Salmonella*-induced enteritis. The bacterial or host target proteins for the HECT Ub ligase activity of SopA may well be involved in PMN migration, but are unknown at this stage. Together, the data suggest that SopA displays multiple functions during *Salmonella* infection.

Yersinia sp. and *Pseudomonas aeruginosa* may also modulate the activity of their effectors by a similar strategy. *Yersinia*, the causal agent of plague (*Y. pestis*) and gastrointestinal disorders (*Y. pseudotuberculosis* and *Y. enterocolitica*), is an extracellular pathogen that injects several effectors through its T3SS to provoke disease [28]. Several of these effectors have been shown to interfere with actin cytoskeleton dynamics that are involved in blocking phagocytosis and subsequent bacterial killing. *Y. pseudotuberculosis* YopE contributes to virulence by inducing depolymerization of actin filaments in the host cells early after contact with *Y. pseudotuberculosis* via the inhibition of Rho GTPases, which control rearrangements of the actin skeleton. This activity also prevents the formation of pores in the host membranes and subsequent host cell death, and thus enables a prolonged colonization of the host [29,30]. In *Y. enterocolitica*-infected cells, YopE is polyubiquitinated on lysine K75 and targeted for proteasome degradation [31]. At this time, it is not clear whether the host-mediated YopE destruction is beneficial to the host’s defense or to *Y. enterocolitica* infection. On the one hand, the degradation products of YopE could be a bacterial antigen source for the host to fend off later infections [31,32]. On the other hand, removing YopE could pave the way for YopT (a cysteine protease [33]) and YopO (a kinase [34]), two other T3SS effectors also targeting actin rearrangements in the host cell. Intriguingly, YopE and *S. typhimurium* SptP both have GTPase-activating protein activity that indirectly inhibits the pathogen-induced actin polymerization, but it is interesting to note that YopE is actively degraded by the host UPS, whereas SptP has a much longer half-life. These two effector proteins, translocated by different pathogens, seem to have evolved a similar strategy for blocking actin polymerization, yet subtle differences in interaction with the host UPS seem to reflect differences in infection strategy.

ExoU is the major T3SS effector of the opportunistic pathogen *Pseudomonas aeruginosa* and is directly responsible for the death of the infected host cell. This effector has a phospholipase activity inside the host cell, and researchers have recently shown that it is targeted to the host cell membrane and ubiquitinated [35]. ExoU undergoes ubiquitination of a specific lysine residue (K178) by an as yet unknown mechanism. No more than two Ub moieties are added onto K178, and those are mostly via the K63 residue of the first Ub. This modification is not responsible for ExoU activity, plasma membrane location, or toxicity, and has only a minor impact on ExoU stability [35]. The latter point is not surprising considering the length and the topology of this ubiquitination event [5,35], but at this point a role for ubiquitination of ExoU, most likely the result of its membrane localization, is not clear. Interestingly, *Y.*



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Figure 3. T3SS Effectors Interfering with Ub Signaling in the Mammalian Innate Immune Response

Ubiquitination plays an important regulatory role in different steps of the innate immune signaling cascades. This simplified representation shows the different levels at which T3SS effectors (black-boxes) thus far have been shown to interfere with the pro-inflammatory host immune response; this interference can be at the signaling node represented by the TAK1 complex (affecting both NF- κ B and the MAPK signaling pathways), or downstream of TAK1, only affecting the NF- κ B pathway. Numbers in brackets indicate the specific references. Details of the immune signaling are given in the text.

enterocolitica YopE also has a specific subcellular targeting to the perinuclear membrane, and this property is determined by the amino acids 54 to 75 [36]. Coincidentally, it is the lysine residue K75 that is subjected to this specific ubiquitination, thus suggesting the possibility of an ubiquitination process associated with membrane localization [31].

Effectors That Interfere with Important Ubiquitination Steps Involved in Mammalian Innate Immune Signaling

The innate immune system is the first line of defense in mammals against microbe infection, and it requires several regulatory ubiquitination steps [37]. Several examples have been published of bacterial T3SS effectors that directly interfere with the ubiquitination level of both K48- and K63-linked poly-Ub chains on mediator proteins in the pathogen-induced host defense signaling cascade, thus allowing the bacterium to undermine a proper innate immune response to promote disease. The host's defense mechanism involves

receptor-mediated signaling via the mitogen-activated protein kinase (MAPK) and the nuclear factor κ B (NF- κ B) pathways (we refer the reader to the following reviews [37–40] and Figure 3). Briefly, one of the ways the host can sense pathogens is via perception of microbial-associated molecular patterns (or MAMPs, such as lipoproteins, methylated DNA, lipoteichoic acid, flagellin, and lipopolysaccharide) by membrane-anchored Toll-like receptors (TLRs). Pro-inflammatory cytokines, such as tumor necrosis factor α (TNF α) and IL-1, are produced as an alert for the immune system via TLR signaling in response to pathogens, and bind to receptor proteins of the tumor necrosis factor receptor (TNFR) and IL-1 receptor (IL-1R) families. Activated TLRs and IL-1Rs recruit downstream adapter and signaling molecules that are involved in the activation of the crucial signal transducer TNFR-associated factor (TRAF) 6, whereas TRAF2 transduces the signal received by TNFR [37,40,41]. TRAF6 acts as an E3 Ub ligase that recruits the E2 enzyme complex Uev1A/Ubc13-

polyubiquitinate itself [42]. This autoubiquitination step allows the recruitment of TGF- β -activated kinase 1 (TAK1)-binding protein (TAB) 2 and TAB3, and subsequently TAK1 and TAB1. After formation of the complex, TRAF6 K63-polyubiquitinates TAK1 [37,38]. Although the precise mechanism is not yet clear, TAB1 also seems to be K63-polyubiquitinated [43]. TAK1 is a highly conserved kinase complex that acts as a central component of the immune response pathway; it can autophosphorylate [42–44] and activate the MAPK (p38, c-Jun NH₂-terminal kinase) pathway by mitogen-activated protein kinase kinase (MEKK) 3 and MEKK6 phosphorylation, as well as the NF- κ B pathway by inhibitor of nuclear factor κ B (I κ B α) kinase (IKK) β phosphorylation [42]. After this latter modification, IKK β could be monoubiquitinated by an as yet unknown mechanism [45]. The IKK γ or NF κ B essential modifier (NEMO) subunit is also K63-polyubiquitinated by TRAF6, which allows the formation of an active IKK complex from the subunits IKK α , IKK β , and IKK γ . The transcriptional activator NF- κ B is sequestered in the cytoplasm by I κ B α and thus is inactive. Phosphorylation of I κ B α by the activated IKK complex allows the recognition of I κ B α by the E3 Ub ligase SCF^{BT₁CP} (Skp1/Cullin1/F-box), which K48-polyubiquitinates I κ B α for proteasome degradation. This provokes the release and subsequent nuclear translocation of NF- κ B, where it acts as a transcription factor of a large array of target genes, including anti-apoptotic and immune responses genes.

Y. pseudotuberculosis and *Y. pestis* YopJ, and their homologue YopP in *Y. enterocolitica*, (phrased YopJ/P when we refer to *Y. pseudotuberculosis* YopJ and *Y. enterocolitica* YopP proteins at the same time) were shown to interfere with the host inflammatory response via both the MAPK and the anti-apoptotic NF- κ B signaling pathways (Figure 3), which prevents the production of pro-inflammatory cytokines such as TNF α and IL-8, and triggers apoptosis. YopJ/P homologues are found in *S. typhimurium* (protein AvrA) and in some plant pathogenic bacteria [46]. These proteins were assigned as C55 cysteine proteases [47], and by sequence comparison were originally hypothesized to be deSUMOylating enzymes, even though no specific substrate could be identified [48,49]. Recently, different research groups have identified YopJ/P as deubiquitinating enzymes directly involved in the inhibition of the inflammatory response [43–45,50]. Zhou and collaborators showed that *Y. pseudotuberculosis* YopJ can deubiquitinate the K63-poly-Ub chain of TRAF6 (and TRAF2) inducing the inhibition of both the MAPK and NF- κ B pathways. *Y. pseudotuberculosis* YopJ was also shown to deubiquitinate the K48-poly-Ub chain from I κ B α , preventing proteasome-mediated degradation of I κ B α and the resulting NF- κ B nuclear translocation [50]. In addition, *Y. enterocolitica* YopP can cleave TRAF6 and NEMO K63-poly-Ub chains, but this activity could only be proven in vitro [44]. Recently, Thiefes and colleagues showed that *Y. pestis* YopP was not able to deubiquitinate TRAF6, but rather that it cleaved the K63-Ub chains of TAK1 and TAB1 [43]. The versatility of *Y. pseudotuberculosis* YopJ was further emphasized by the finding that it could remove the single Ub linked to IKK β [45]. Tampering with Ub in the inflammatory pathway is achieved so efficiently by YopJ of *Y. pseudotuberculosis* and Yop P of *Y. enterocolitica* that *Y. pestis* has evolved a less invasive strategy. Indeed, *Y. pestis* YopJ is believed to be injected by the T3SS less efficiently than YopP, and, as a consequence, does not

result in fast apoptosis of macrophages, but rather weakens the inflammatory response, enabling the pathogen to be transported within the macrophages to target organs [51]. These reports emphasize the crucial role of bacterial effectors in the deubiquitination of key components in the MAPK and NF- κ B pathways, on both K48- and K63-poly-Ub chains (Figure 3). Interestingly, the eukaryotic deubiquitinating enzymes cylindromatosis tumor suppressor protein and A20, which are required for cell homeostasis [52–54], can also deubiquitinate the K63-polyubiquitin chains of signaling intermediates of the NF- κ B pathway, but are not active on the K48 chains [50,55]. This illustrates that pathogens clearly use mechanisms that are familiar to the host. Even though YopJ/P seem to have an extended deubiquitinating activity (both of K48- and K63-poly-Ub chains), these proteins don't seem to be able to cleave SUMOylated proteins. Indeed, YopJ/P are deubiquitinating enzymes in vitro, with no activity on SUMOylated proteins [50]. The cysteine residue (C172) that was originally defined for the deSUMOylation activity [49] is also required for the deubiquitinating activity [44,50]. We would like to add that recent work shows that *Y.*

pseudotuberculosis YopJ blocks signaling by binding and acetylating residues in the activation loop of MEKK6 (a mitogen-activated protein kinase kinase upstream of c-Jun NH₂-terminal kinase and p38) [56]. The authors hypothesize that a similar activity could prevent phosphorylation of IKK β , thus preventing further activation of the NF- κ B pathway [56].

Evidently more work is necessary to unravel the mode of action of the YopJ/P family of T3SS effectors. It would be interesting to re-evaluate the function of YopJ/P homologues widely present in plant pathogenic bacteria and currently identified as deSUMOylating enzymes [46,57–59].

The facultative intracellular pathogen *Shigella flexneri*, causal agent of shigellosis in humans, has among its repertoire of T3SS effectors a protein called OspG that is structurally related to a kinase. This protein was shown to be injected into epithelial cells, where it weakens the host innate immune response [60]. OspG negatively regulates the NF- κ B inflammatory response by interfering with the proteasome-dependent degradation of I κ B α . OspG binds and inhibits ubiquitinated E2 Ub-conjugating enzymes: it interacts with UbcH5, which is necessary for the Ub supply to the E3 Ub ligase SCF^{BT₁CP}, the specific SCF complex that controls I κ B α degradation (Figure 3). OspG displays a kinase activity inducing its autophosphorylation. This activity is necessary for the function of OspG, but doesn't seem to be involved in the phosphorylation of I κ B α prior to its K48-polyubiquitination and proteasome-mediated degradation. The mechanism by which OspG inhibits the SCF^{BT₁CP}-mediated I κ B α degradation still has to be elucidated. This T3SS effector is injected into host cells during the early stages of infection [61]. An attractive hypothesis is that the inhibition of the NF- κ B inflammatory response in these early stages facilitates cellular colonization by a limited number of luminal bacteria [60]. Considering that OspG can interact with different E2 Ub-conjugating enzymes, this effector might interfere with other UPS targets in the host cells as well.

Non-pathogenic or attenuated *Salmonella* strains (*S. typhimurium* PhoPc mutant [62,63] and *S. pullorum*, a poultry-specific strain) have also been shown to attenuate the NF- κ B-mediated inflammatory response [64]. In doing so, these organisms can thrive in the intestinal microflora. The

mechanism by which these strains inhibit the inflammatory response seems to involve the reduction of Cullin1 neddylation. Non-neddylated Cullin1 is still capable of taking part in the SCF^{βTrCP} E3 Ub ligase complex, but could be impaired in the recruitment of the E2 Ub-conjugating enzyme [65,66]. The absence of a functional SCF^{βTrCP} complex would then result in the absence of ubiquitination of phospho-IκBα, hence explaining the observed stabilization of phospho-IκBα. As pointed out by the authors, the bacterial factors responsible for the attenuation of the Cullin1 neddylation haven't been identified yet.

Effectors Involved in Ubiquitination of Host Proteins to Suppress Plant Innate Immune Responses

Recent work has revealed striking similarities in the response between animals and plants in the recognition of MAMPs, as illustrated by the discovery of a plant receptor reminiscent of TLR5 in humans [67,68], as well as in downstream antimicrobial defense responses that are signaled via MAPK cascades and induction of target gene expression [69,70]. MAMP recognition can initiate basal defense responses, such as strengthening of the cell wall by callose deposition [71,72]. Specific bacterial T3SS effectors are capable of suppressing this basal defense mechanism [73–76]. Another type of resistance, specific for plants, is the hypersensitive response (HR), which causes rapid cell death at the site of infection on resistant plants and thereby prevents bacterial multiplication and spread. This type of resistance is induced by specific recognition of bacterial virulence factors (including T3SS effectors) by cultivar-specific resistance proteins [75]. Also, in the case of HR-mediated resistance, some bacteria have evolved T3SS effectors capable of avoiding this specific type of induced resistance [71,72,75,77–81]. Below, we present two *P. syringae* T3SS effectors that are capable of suppressing different layers of plant defense, probably by controlling the ubiquitination and degradation of specific proteins by the cell UPS.

P. syringae causes bacterial speck disease on susceptible plants. HopM1 is a *P. syringae* T3SS effector required for virulence and is known to suppress the plant host cell wall-associated defense [72]. Recently, Nomura and colleagues showed that during bacterial infection of *Arabidopsis* plants, HopM1 mediates the proteasome-dependent elimination of AtMIN7, a plant protein involved in cell wall-associated host defense [78]. Interestingly, HopM1 has no classical E3 Ub ligase features, leading to the hypothesis that HopM1 may act as an adaptor protein mediating the recognition of AtMIN7 by the plant UPS. AtMIN7 is a GEF of the adenosine diphosphate ribosylation factor (ARF) subfamily. ARF-GEFs are important for vesicle trafficking by activation of Ras-like small GTPases [82]. Several lines of evidence show that vesicle trafficking plays an important role in plant immunity [72,83–85]. When challenged with a ΔCEL *P. syringae* mutant strain (lacking the *hopM1* gene), AtMIN7 knock-out plants accumulate less callose deposits and are more susceptible to infection than wild-type plants. Altogether, the data suggest that the role of HopM1 in virulence is to inhibit vesicle trafficking associated with cell wall-associated host defense by targeting AtMIN7 for degradation by the host UPS [78].

The *P. syringae* pv *tomato* (strain DC3000) T3SS effectors

AvrPto and AvrPtoB both elicit an HR response in tomato plants expressing the *Pto* resistance gene. [86,87]. This rapidly induced localized programmed cell death at the site of infection enables plants to resist colonization by the pathogen. Interestingly, AvrPtoB is also capable of suppressing programmed cell death induced by the AvrPto/Pto recognition in *Nicotiana benthamiana*, and HR elicited by other bacterial T3SS effectors, fungi-specific HR-inducing protein, and even the pre-apoptotic mouse protein Bax [88,89]. AvrPtoB is a modular protein with an N-terminal part that induces HR-related cell death and a C-terminal portion that controls cell death suppression [90]. The C-terminal domain was recently shown to possess the structural features of a RING U-box type E3 Ub ligase [81]. This domain, as well as full-length AvrPtoB, indeed functions as an active E3 Ub ligase capable of autoubiquitination [79,81] and possibly of ubiquitination of plant substrates [79]. The E3 Ub ligase activity is functionally important since mutations impairing the recruitment of the E2 Ub-conjugating enzyme or the autoubiquitination prevent both cell death suppression activity and full virulence of strain DC3000 [79,81]. Even though no target has yet been identified, a possible explanation for the mode of action of AvrPtoB is the specific recognition, ubiquitination, and proteasome-dependent degradation of plant cell death positive regulators. It should be noted here that, in a recent report, AvrPto and AvrPtoB have been identified as potent and early suppressors of MAMP-induced MAPK-dependent innate immunity pathway in *Arabidopsis*, but this function of AvrPtoB is not affected by a mutation that disrupts the E3 Ub ligase activity [74].

Effectors Mimicking Host E3 Ub Ligases

Mimicking eukaryotic proteins appears to be a strategy commonly used by pathogenic bacteria to promote virulence [91,92]. This can be achieved by convergent evolution, which “produces” a new effector protein with structural characteristics enabling the functional mimicry of a host protein (e.g., AvrPtoB). But this can also be achieved by a more “opportunistic” scenario, in which the bacterial pathogen or one of its ancestors has acquired genetic material by lateral transfer and then maintains and adapts functional domains according to their selective advantage in virulence. The two examples discussed below illustrate the latter scenario and highlight a subtle mechanism bacteria have evolved to directly interfere with plant functions via the UPS.

Agrobacterium tumefaciens causes crown gall disease on a broad range of plants. The bacterium uses a type IV secretion system (T4SS) not only to translocate effectors into eukaryotic cells, but also to mediate the transfer of a single-stranded DNA molecule (transferred [T]-DNA), resulting in genetic colonization of the host [93–95]. VirF is a T4SS effector that determines host range and is necessary for full virulence on certain host plants [96,97]. VirF was the first prokaryotic protein shown to contain a conserved F-box domain [98]. F-box proteins (FBPs) are key components of the SCF type E3 Ub ligase complex, because they recruit the target protein for destruction by the 26S proteasome. Through the F-box domain, FBPs interact with the SKP1 component of the E3 Ub ligase complex [99]. The *Arabidopsis* homologues of the yeast SKP1 protein, ASK1 and ASK2, were

isolated as interactors of VirF [98]. The F-box of VirF was shown to be essential not only for this interaction in vitro, but also for virulence. To determine the precise role of VirF in the infection process, recent studies are aimed at identifying the target proteins destined for ubiquitination and possibly degradation by the proteasome. Recently, it was shown that VirF interacts with the plant protein VirE2-interacting protein 1 (VIP1), which leads to degradation of VIP1 and, indirectly, of the effector protein VirE2 [100]. VirE2 is a single-stranded DNA-binding protein that is transported independently from the T-DNA by the T4SS into the host cell [101,102], where it cooperatively binds the T-DNA to facilitate nuclear uptake [103,104] and protect it from degradation [105]. VirE2 contains functional nuclear localization signals [104,106], but these signals overlap with the DNA-binding domain [106,107], which make it difficult to show an in vivo function of the nuclear localization signal region in nuclear uptake of the T-complex. The *Arabidopsis* protein VIP1 was identified as an interactor of VirE2 [108]. This protein was shown to interact with karyopherin- α , a member of the importin family involved in nuclear import of proteins via recognition of their nuclear localization signals. Citovsky and colleagues suggested a role for VIP1 in *A. tumefaciens* infection as a molecular adaptor between VirE2 and karyopherin- α that results in nuclear uptake of the T-complex [109]. Recently, Tzfira and colleagues proposed that VirF, which binds to VIP1 but not VirE2, is involved in the nuclear proteasome-dependent degradation of VIP1 and indirectly in that of VirE2, and may thus play a role in uncoating the T-complex from VirE2 molecules prior to integration of the T-DNA in the host genome [100]. A host-dependent role in virulence for VirF by indirectly targeting another effector protein for degradation is intriguing; yet, it remains to be determined whether VirF is able to destabilize VIP1 and VirE2 when complexed with T-DNA and, more importantly, during infection. In preliminary experiments, using the C-terminal part of VirF (lacking the F-box domain) as bait in a yeast two hybrid screen, several *Arabidopsis* proteins have been identified as putative targets of VirF (E. Jurado-Jácome, P. Hooykaas, and A. Vergunst, unpublished data). Among these are proteins that have been shown to be involved in host defense-related processes. Although the interaction of some of these proteins has been confirmed in vitro, the relevance of these interactions during infection and their VirF-mediated degradation remains to be confirmed. As described above, a function in disarming host proteins involved in defense against bacterial attack and suppression of the immune response seems to be a general mode of action for effectors of both mammalian and plant pathogens.

The plant pathogen *Ralstonia solanacearum* uses a T3SS to promote “bacterial wilt” on a variety of plant hosts [110–112]. Among the large repertoire of T3SS effectors identified in this bacterium [110–112] is a family of proteins that is likely to function as eukaryotic FBPs [113]. Indeed, each of the seven members of this effector family harbors both an N-terminal F-box motif for interaction with other subunits of the E3 Ub ligase complex, and a long leucine rich repeat (LRR) domain. A characteristic feature of the LRR is the presence in each of the 24 amino acid-long repeats of conserved residues forming the motif GAXALA, hence the name “GALA” proteins. The structure of these T3SS effector proteins is highly similar to the LRR subclass of plant FBPs

[114]. We further showed that GALAs are capable of interacting with several of the 19 *Arabidopsis* SKP1-like proteins (ASKs). Like *A. tumefaciens* VirF, GALAs interact with ASK1 and ASK2, but also interact with other ASKs, in a manner that is reminiscent of plant FBPs [114,115]. Pathogenicity tests revealed that none of the individual GALA effectors is indispensable for virulence of *R. solanacearum* on *Arabidopsis* or tomato [112]. The finding that a strain deleted of all seven GALA genes is significantly less virulent on tomato and *Arabidopsis* [113] suggests that two or more non-functionally overlapping GALAs are required. Interestingly, when tested on *Medicago truncatula*, another host plant, a single mutant for the GALA7 gene appears dramatically affected in its virulence. The virulence capacity of this single mutant is restored by complementation with a full length GALA7 construct, but not by a GALA7 gene construct deleted of its F-box domain. These results support a model, similar to VirF, in which specific GALAs (GALA7 on *Medicago truncatula*) and combinations of GALAs could form bacterium/plant composite SCF-type E3 Ub ligases in specific host cells, possibly to ubiquitinate and subsequently degrade mediator(s) of plant defenses.

Only the Tip of the Iceberg

The bacterial plant pathogens *A. tumefaciens* and recently *R. solanacearum* were the first prokaryotes shown to harbor proteins with an F-box that is essential for virulence [98,113]. These FBPs are substrates of T4SS and T3SS, respectively [98,113]. The F-box-containing protein Msi061 of the plant symbiont *Mesorhizobium loti* was also demonstrated to be transported into plant cells in a heterologous translocation assay by the *A. tumefaciens* T4SS [116]. The recent completion of the genome sequences of the *Legionella pneumophila* strains Paris and Lens enabled the annotators to identify three genes that likely encode FBPs, and one gene that encodes a protein with two U-box domains [117], making these proteins attractive candidates for participating in E3 Ub ligases within eukaryotic host cells. The complete genome sequence of many bacteria, including human and plant pathogens, and other bacteria that have close associations with eukaryotes during their life cycle, are now available. Our curiosity about the extent of bacterial effector candidates exploiting the host UPS made us mine the most recent protein database release using the protein domain signature search tool available at InterPro (<http://www.ebi.ac.uk/interpro>, data release 13.0). We searched for the eukaryotic-specific putative E3 Ub ligase U-box (IPR003613) and F-box (IPR001810) motifs. In addition to the *L. pneumophila* U-box protein Lpp2887 that was recently annotated [117], we identified one gene encoding a putative U-box domain (locus pc1652) in *Candidatus Protochlamydia amoebophila* UWE25, an obligate endosymbiont of free-living amoebae [118]. In contrast, the search for F-box domains encoded by bacterial genomes was more productive and yielded several new candidates (Table 2). Although InterPro identified the *A. tumefaciens* VirF protein [98], it only found three out of the seven *R. solanacearum* GALA proteins [113], and did not detect the *Mesorhizobium loti* msi061 protein [116], indicating that our screen was not saturating. In *L. pneumophila*, however, our analysis found not only the three already annotated FBPs [117,119], but also three additional FBP candidates (Table 2). *Coxiella burnetii*, the agent of Q-fever

Table 2. Bacterial F-Box-Containing Proteins

Bacterium Class	Bacterium	F-Box (IPR001810)-Containing Protein			Other IPR Matches			Orthologues	Reference
		Name	UniProtKB Accession	Size (aa)	F-Box Position				
Chlamydiae	<i>Candidatus Protochlamydia amoebophila</i> strain UWE25	Q6M9Q3_PARUW		647	1–31, 50–98	IPR011046 (WD40), IPR001680 (WD40), IPR011044 (quinoprotein amine dehydrogenase)			
		Q6M9S9_PARUW		462	6–55	IPR011046 (WD40)			
		Q6MA59_PARUW		732	124–175	IPR013101 (LRR)			
		Q6MB76_PARUW		697	25–74	IPR002110 (Ankyrin)			
		Q6MBH1_PARUW		469	6–55	IPR011046 (WD40)			
		Q6MBW8_PARUW		467	6–55	IPR011044 (quinoprotein amine dehydrogenase)			
		Q6MBY1_PARUW		1681	7–46	IPR003323 (ovarian tumor)			
		Q6MD27_PARUW		73	9–56				
		Q6MD91_PARUW		948	24–71	IPR011046 (WD40), IPR001680 (WD40), IPR001646 (pentapeptide repeat)			
		Q6MD92_PARUW		941	24–71	IPR001680 (WD40), IPR001646 (pentapeptide repeat), IPR011042 (TolB, C-terminal)			
α -proteobacteria	<i>Agrobacterium tumefaciens</i> strain 15955	Q6MD96_PARUW		915	24–71	IPR011046 (WD40), IPR001680 (WD40), IPR001646 (pentapeptide repeat)		O52656_AGRVI	[98]
		VirF	VIRE_AGR79	202	21–75	nd			[116]
		Q8KGY7_RHIL0		287	nd	IPR001611 (LRR)			[113]
		GALA1	Q8XRE0_RALSO	661	61–108	nd			[113]
		GALA2	Q8X509_RALSO	1035	nd	nd			[113]
		GALA3	Q8XT56_RALSO	603	nd	nd			[113]
		GALA4	Q8XYF8_RALSO	462	nd	nd			[113]
		GALA5	Q8XYF7_RALSO	538	62–109	IPR001611 (LRR)			[113]
		GALA6	Q8XZN9_RALSO	620	nd	nd			[113]
		GALA7	Q8XZN8_RALSO	647	120–170	IPR001611 (LRR)			[113]
γ -proteobacteria	<i>Pseudomonas syringae</i> pv <i>phaseolicola</i> strain 1448A	Q48MIF1_PSE14		355	175–223			Q4ZLS5_PSEU2, Q87UI5_PSESM	
		Q38XH6_XANC5		450	45–92	IPR006311 (twin-arginine translocation pathway signal)		Q8PPD2_XANAC	
		Q5X2A7_LEGPA		212	20–68				[117]
		Q5Z5J2_LEGPH		444	6–55	IPR002110 (ankyrin)			
		Q5ZTD8_LEGPH		593	17–67	IPR009091, IPR00040 (regulator of chromosome condensation)			
		Q5ZTL7_LEGPH		188	3–51	IPR002110 (ankyrin)		Q5WU05_LEGPL, Q5X3F4_LEGPA	[117]
		Q5ZVN2_LEGPH		383	13–60	IPR011009 (protein kinase-like), IPR002573 (choline/ethanolamine kinase)		Q5WU00_LEGPL, Q5X5F7_LEGPA	
		Q5ZZ40_LEGPH		188	9–57			Q5WZZ5_LEGPL, Q5X8L7_LEGPA	[117]
		Q83A11_COXBU		77	18–65	IPR009091 (regulator of chromosome condensation)			
		Q83DC4_COXBU		246	13–60				
<i>Coxiella burnetii</i>	<i>Coxiella burnetii</i>	Q83EG7_COXBU		357	90–137	IPR002110 (ankyrin)			

The search was performed using the InterPro (or IPR) database (<http://www.ebi.ac.uk/interpro>) data release 13.0, using the F-box-defined Hidden Markov-Model, IPR001810. Orthologues (amino acid identity higher than 80%) were not considered as separate entries. aa, length of the proteins in amino acids; nd, not determined (entries which were not detected by InterPro). doi:10.1371/journal.ppat.0030003.t002

[120] closely related to *L. pneumophila*, also contains three genes encoding putative FBPs (Table 2). *Candidatus Protochlamydia amoebophila* UWE25 [118] has numerous (11) proteins containing putative F-boxes. This bacterium is a recently characterized relative of pathogenic *Chlamydia*, but has a much larger genome size, indicating that massive reorganization and genome reduction took place in *Chlamydia* sp. after divergence in pathogenic and symbiotic *Chlamydia* [118]. The absence of any detectable FBP in pathogenic *Chlamydia* sp. might be the result of this genome reduction, and the large number of FBPs in UWE25 possibly originate from ancient lateral transfer [118]. This suggests a greater importance of these putative FBPs in symbiosis than in the control of the virulence of its pathogenic relative.

Our screen also revealed conserved F-box-encoding genes among several sequenced plant pathogens, including *Xanthomonas* sp., and several *P. syringae* pathovars. In summary, our in silico analysis indicates a wide range of bacteria, from the class of Chlamydiae to α -, β - and γ -proteobacteria, predicted to contain putative FBPs. It will be interesting to find out whether the bacteria with putative FBPs can indeed inject these proteins as substrates of the T3/4SS into host cells, where they could interfere with UPS-controlled mechanisms to benefit in the survival of the pathogen.

Our purpose in this review was to illustrate the different mechanisms used by bacteria to explore the host's UPS by their T3SS or T4SS effectors. To put the exploitation of the UPS by bacterial effectors of T3/4SS into perspective with other mechanisms used by pathogens to interfere with their host's UPS, we would like to just touch on several rapidly expanding groups. Bacterial toxins, transported into host cells by mechanisms other than T3/4SS, have been shown to interfere with the host UPS. Inside the host cell, the subcellular localization of *Listeria monocytogenes* listeriolysin O and phospholipase C are partially controlled by the host UPS machinery [121,122]. The *Escherichia coli* cytotoxic necrotizing factor-1 toxin induces the permanent activation of host Rho proteins by locking them in a GTP-bound state. These Rho proteins are then rapidly ubiquitinated and degraded by the host cell [123–125]. The overall effect is an increase in Rho activity followed by Rho depletion, resulting in an efficient bacterial internalization and a weaker host inflammatory response [126]. *Rickettsia conorii*, an obligate intracellular pathogen, also seems to require its host UPS for contact-mediated internalisation [127]. Viruses are well known for their ability to subvert their host UPS either by regulating, or by mimicking, host UPS subunits [128–130]. Recent studies suggest that eukaryotic pathogens can also deliver proteins that interfere with the UPS in host cells. Indeed, thanks to a feeding stylet, plant parasitic nematodes can deliver gland-secreted ubiquitin extension proteins potentially interfering with the Ub pathway in plant cells [131,132].

Conclusion

A number of animal and plant pathogenic bacteria have evolved type III and type IV effectors that, once translocated into the host cell, have the capacity to interfere directly with ubiquitin signaling, a mechanism fundamental to the eukaryotic host cell. These bacteria have developed different strategies to exploit the host cell ubiquitin/proteasome system to their advantage to (i) control the timing of action of their

virulence effectors, as exemplified by the *Salmonella* sp. effectors SopA and SptP, (ii) target specific signaling intermediates involved in mammal or plant innate immunity, as performed by *Yersinia* sp. YopJ/P, or (iii) mimic specific host-like UPS components, illustrated by the bacterial FBPs.

The variety of examples presented in this review illustrates the effectiveness of pathogens in interfering with host Ub signaling pathways. It also illustrates that each bacterium has developed a different mode of interference with the UPS by its effectors, depending on its infection strategy. Some bacteria suppress their host's immune response (inhibition of innate immune signaling, inhibition of vesicle trafficking), whereas others control the host cell actin cytoskeleton for bacterial internalization. This exciting area of research is advancing at high speed; future research will certainly result in the discovery of more effectors that interfere with the UPS and the identification of specific host targets and the host functions affected. Such discoveries will supplement research on other (eukaryotic and prokaryotic) pathogens and on mechanisms other than T3/4SS that bacteria have evolved to interfere with the host's UPS. It will undoubtedly result in a better understanding of this intimate host–pathogen interaction, as well as provide new insights in eukaryotic ubiquitination processes. In addition, it may also form the basis for the development of a specific new class of antimicrobials. ■

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