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Ubiquitin, SUMO and Nedd8 ; Critical Targets of Bacterial Pathogens

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Abstract

Manipulation of host protein post-translational modifications is used by various pathogens to interfere with host cell functions. Among these modifications, ubiquitin and ubiquitin-like proteins constitute criticial targets as they are regulators of pathways essential for the host cell. In particular, these post-translational modifiers control pathways that have been described as critical for infection such as pathogen entry, replication, propagation or detection by the host. Although bacterial pathogens lack ubiquitin or ubiquitin-like protein systems, many of them produce proteins that specifically interfere with these host post-translational modifications during infection. In this review, we will discuss the different mechanisms used by bacteria to interfere with host ubiquitin and the two ubiquitin-like proteins (UBLs) SUMO and NEDD8.

Highlights :

* Ubiquitin and UBLs regulate essential pathways of the host cell involved in critical steps of bacterial infections. Not surprisingly, bacterial pathogens have evolved multiple strategies to interfere with these host post-translational modifications.

* Besides Ubiquitin, UBLs such as SUMO and NEDD8 have recently emerged as prominent targets of bacterial pathogens.

* Strategies used by bacteria to interfere with host Ubi/UBL encompass the targeting of Ubi/UBL conjugation machineries, the modulation of the Ubi/UBL conjugation level of specific host factors and the direct targeting of Ubi/UBL proteins.

* Host proteins modified by Ubi/UBL and targeted by bacteria cluster into specific host cell functions such as gene regulation, cytoskeleton dynamics and cell-autonomous immunity.

* Bacteria hijack the host Ubi/UBL systems to modify their own proteins allowing a regulation of their intracellular localization, stability or interaction abilities.

Outstanding questions :

* Are the recently described non-canonical ubiquitination mechanisms (*i.e.* conjugation involving non-RING/non-HECT E3 ligases or E1/E2-independent ubiquitin conjugation) strictly restricted to bacteria? Or are there functional homologs of these bacterial enzymes encoded by human cells?

* Recent improvements in proteomic analyses now allow to thoroughly monitor changes in the host ubiquitinome/"UBL-ome" in response to infection. These approaches usually provide thousands of putative candidate proteins showing altered Ubi/UBL-conjugation level in response to infection. Which strategies researchers should use to cope with this complex set of data and identify the key players affecting the outcome of infection ?

* What are the mutations in the human population affecting the Ubi/UBL systems that may confer higher susceptibility to bacterial pathogens ?

* Would drugs targeting bacteria-specific enzymes that interfere with host Ubi/UBL conjugation be efficient to treat infectious diseases ?

Ubiquitin and Ubiquitin-like proteins constitute essential modifiers of host proteins

Post-translational modification (PTM) of proteins are involved in the regulation of protein stability, activity, localization or interactions with other cellular components. PTMs encompasses a wide range of chemical processes. They include the cleavage of peptide bonds (proteolysis), the modification of specific amino acid side chains such as deamidation (*i.e.* the removal of an NH_3 group) or eliminylation (*i.e.* the irreversible removal of a phosphate group), and the covalent addition of chemical moieties ranging from simple groups (such as phosphate, acetyl or methyl groups) to more complex groups such as sugar, lipids or even small polypeptides [1,2].

The human genome encodes several polypeptides that can be covalently linked, via their C-terminal glycine residues, to target proteins. The best-studied of these polypeptides is ubiquitin (Ubi), a small polypeptide of 76 amino acids discovered more than 40 years ago [3-5]. Besides Ubiquitin, other polypeptides such as SUMO (Small Ubiquitin-like Modifier) [6], NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) [7], ISG15 (interferon-stimulated gene 15) [8] or FAT10 (HLA-F-adjacent transcript 10) [9] can be similarly conjugated to target proteins. These polypeptides are grouped in the so-called ubiquitin-like protein (UBL) family and share high structural homology with ubiquitin [10].

The consequences of Ubi/UBL conjugation on the fate of the modified proteins are very diverse. Ubi/UBL can alter the half-life of the modified proteins, for example by targeting them to proteasomal degradation. They can change the targets' structure, thereby affecting their catalytic activity. They can add new surfaces of interactions or mask internal binding domains and change the targets' interactome. In particular, the cell encodes many "receptors" containing Ubiquitin-binding domains (UBDs) or UBL binding domains (such as the SUMO interacting motifs [SIMs]), that interact with proteins once conjugated to Ubi/UBL and "decode" these

88 modifications into biochemical cascades in the cell [6,11]. Besides the well-known example of
89 K48-Ubiquitin chain conjugation (see below), that addresses modified proteins to proteasomal
90 degradation, it is usually difficult to anticipate the consequences of Ubiquitin or UBL
91 conjugation to a given protein.
92 Ubi/UBL are essential regulators of fundamental pathways in cell biology. Some of these
93 pathways are critical for the outcome of infection by pathogens. For example, Ubiquitin is a
94 major regulator of the NF-κB pathway, that triggers the expression of proinflammatory
95 cytokines in response to pathogen detection [12]. SUMO is a central player in the regulation of
96 type I interferon and in anti-viral gene expression programs [13]. ISG15 plays several
97 independent roles in anti-viral defense and can restrict intracellular bacteria replication *in vitro*
98 and *in vivo* [8,14,15]. FAT10 has been reported to be involved in xenophagy and in
99 antimicrobial defense [9,16]. It is thus not surprising that pathogens have evolved strategies to
100 target Ubi/UBL and interfere with these different cellular processes.
101 In this review, we will present how pathogens interfere with the host Ubi/UBL systems.
102 Ubiquitin and UBL systems have been shown to be targeted by diverse pathogens such as
103 viruses, bacteria or parasites, including *Plasmodium falciparum* or *Toxoplasma gondii* [17-24].
104 We will focus on pathogenic bacteria as they display the widest variety of Ubi/UBL interfering
105 strategies known to date. Although bacteria do not have their own Ubi/UBL systems, numerous
106 species encode virulence factors that actually manipulate host Ubi/UBL systems (Table 1).
107 These factors can be toxins, secreted into the extracellular space in the vicinity of the host cell,
108 or effectors delivered directly into host cells via specialized secretion systems such as Type III
109 secretion systems (T3SS) [25,26]. We discuss how bacterial pathogens (i) target Ubi/UBLs
110 conjugation machineries, (ii) increase or decrease the Ubi/UBL conjugation on specific host
111 factors, (iii) directly target Ubi/UBL polypeptides, or (iv) use host Ubi/UBL to modify their
112 own proteins. We bring to light how these mechanisms allow bacterial pathogens to manipulate

specific host cellular pathways in order to promote infection. Understanding how pathogens manipulate host Ubi/UBL pathways is essential as it represents a prominent facet of host-pathogen interactions and may lead to the identification of new therapeutic targets.

Ubiquitin/UBL conjugation mechanisms in eukaryotes

Ubiquitination, *i.e.* the conjugation of ubiquitin, usually occurs on lysine residues of target proteins although conjugation to other amino acids such as threonine, serine, or cysteine may also occur [5]. Ubiquitin itself contains seven lysines (K6, K11, K27, K29, K33, K48 and K63) that can serve as sites for additional cycles of ubiquitin attachment, resulting in the formation of ubiquitin chains. The topology of these chains is diverse, ranging from "homotypic" K48- or K63-linked chains, composed of only one type of ubiquitin linkage, to "mixed" chains containing for example both K11 and K63 linkages [4,27]. An additional type of chain, called a "linear" chain, is generated when ubiquitin is attached to the N-terminus of a second ubiquitin [28]. Targeting of a given protein by ubiquitin may thus result in mono-ubiquitination, multi-mono-ubiquitination (*i.e.* several mono-ubiquitinations on different amino acids) or poly-ubiquitination. Ubiquitin is attached to substrates by a three-step enzymatic cascade involving E1 (Ubiquitin activating enzyme), E2 (Ubiquitin-conjugating enzyme) and E3 (Ubiquitin ligase) enzymes [4]. Ubiquitin is generally first activated in an ATP-dependent manner by E1, which links the C-terminal glycine residue of Ubiquitin via a thioester bond to a cysteine residue within the E1 active site. This activated Ubiquitin is then transferred to the catalytic cysteine residue of an E2 enzyme. E3 ligases then finally mediate the transfer of ubiquitin from the E2 enzyme to specific substrates. There are two major classes of E3s: the HECT (homologous to the E6-AP carboxyl terminus) type and the RING (really interesting new genes)/U-box type. HECT-type E3 Ubiquitin ligases form a reactive intermediate with ubiquitin before its transfer to the substrate protein whereas RING/U-box-type E3 ligases mediate transfer of ubiquitin from

the E2 directly to the substrate protein, without formation of an E3-ubiquitin intermediate [29]. Of note, U-box protein E3 ligases display unique preferences for E2 and ubiquitin chain formation compared to RING E3 ligases and may be classified as an independent type of E3 [30]. In addition to HECT and RING/U-box E3 ligases, other classes of host E3 ligases have been described such as RBR (RING-between-RING) and RCR (RING-Cys-relay) ligases [31,32]. RBR ligases, such as Parkin and HOIP, combine mechanistic features of RING and HECT-type E3 ligases [31], whereas RCR exhibits esterification activity and intrinsic selectivity for non lysine residues [32]. Conjugation of ubiquitin is a reversible process as several cellular isopeptidases (called deubiquitinases or DUBs) can cleave the covalent bond between ubiquitin and its targets and thereby remove ubiquitin [33].

The mechanisms of UBL conjugation on target substrates is similar to that observed for ubiquitination. The enzymes required for all these modifications (*i.e.* E1 UBL activating enzymes, E2 UBL conjugating enzymes and E3 UBL ligases) share highly conserved domain structures [10]. Of note, the number of UBL specific E1, E2 and E3 enzymes is usually smaller than for ubiquitin. For example, SUMO conjugation to thousands of cellular targets seems to rely only on one single SUMO E1 enzyme (SAE1/UBA2), one single SUMO E2 enzyme (UBC9) and a dozen SUMO E3 ligases [6]. Similar to ubiquitin, the formation of UBL chains (where UBLs are conjugated to internal lysines of other UBLs) has been reported for SUMO and NEDD8 [6,7]. Finally, as for Ubiquitin, the host cell encodes several ULPs (UBL-specific proteases) that guarantee the reversibility of UBLs conjugation [6-9].

Harnessing of host Ubiquitin and UBL conjugation by bacterial pathogens

Targeting of host Ubiquitin and UBL conjugation machinery enzymes

Targeting of host E1, E2 or E3 ubiquitin enzymes constitutes a first strategy used by pathogens to dampen ubiquitination (Fig. 1, Key figure). This strategy is used for example by *Shigella*

flexneri, the etiological agent of bacillary dysentery. This bacterium secretes through its T3SS, an effector, named OspI, that deamidates the glutamine residue at position 100 in the human E2 ubiquitin enzyme UBC13 [34]. This deamidation inactivates UBC13 ubiquitin-conjugating activity, leading to the dampening of the ubiquitin-dependent TRAF6-mediated signaling pathway, which is involved in the activation of the NF-κB pathway (see below). This finally results in the inhibition of host inflammatory responses during infection [34]. Extracellular pathogens such as enteropathogenic *Escherichia coli* (EPEC) also target the host ubiquitin conjugation machinery. Adhesion of these bacteria to human cells leads to the degradation of UBE1 and UBA6, the two E1 ubiquitin enzymes [35]. This degradation involves aspartyl protease-dependent and proteasome-independent mechanisms and triggers a global decrease of host protein ubiquitination [35].

The SUMO conjugation machinery constitutes another target for bacterial pathogens. *Listeria monocytogenes*, the bacterium responsible for human listeriosis, dampens SUMOylation of specific host factors by triggering the degradation of UBC9, the unique host E2 SUMO enzyme [25,36,37]. The degradation of UBC9 is triggered by the formation of pores in the host plasma membrane by the bacterial toxin Listeriolysin O (LLO) [25]. As LLO pores are not reported to affect the activity of host deSUMOylases, UBC9 degradation ultimately results in a shift in the SUMOylation/deSUMOylation equilibrium in the cell and the deSUMOylation of host proteins [25]. The deSUMOylation events triggered by LLO were shown to promote *Listeria* infection. Indeed, removal of SUMO from host factors critical for infection, such as specific transcription factors, may modulate their activity and favor bacterial replication or survival in host cells [25,36]. Of note, other toxins of the same family as LLO, and secreted by extracellular pathogens, were shown to downregulate UBC9, indicating that interference with host SUMOylation is a strategy conserved between different classes of pathogenic bacteria [25]. Inhibition of the SUMOylation machinery has also been observed

188 during infection with *Salmonella* Typhimurium, a bacterium responsible for gastroenteritis in
189 humans, and with *Shigella flexneri* but the underlying mechanisms involved here do not rely
190 on the production of bacterial toxins. In the case of *Salmonella* Typhimurium, infection leads
191 to upregulation in the host cell of two small noncoding RNAs (miR30c and miR30e) that
192 downregulate the UBC9 levels [38]. In the case of *Shigella flexneri*, infection is associated with
193 an influx of calcium into the host cell. This ion flux activates the host proteases calpains, which
194 cleave UBA2, one of the two components of the E1 SUMO enzyme [39]. The resulting
195 inhibition of SUMOylation is associated with an increase in *Shigella* entry [39,40].
196 Interestingly, the *Listeria* LLO toxin was recently shown to decrease the level of various E2
197 conjugases, in addition to UBC9, such as Ubiquitin E2s or the NEDD8 E2 UBC12/UBE2M
198 [41]. This suggests that several host Ubi/UBLs systems may be simultaneously altered in
199 response to *Listeria* infection.
200
201 *Secretion of bacterial effectors mimicking host Ubiquitin- and UBL- enzymes*
202 Besides interfering with ubiquitin or UBL-conjugation machineries, bacterial pathogens
203 produce proteins that can replace or act as components of these machineries (Fig. 1). In
204 particular, several bacterial effectors possess ubiquitin E3-like activity (Table 1). Some of these
205 bacterial effectors share structural homologies with the two major types of eukaryotic E3
206 ligases, *i.e.* the HECT type and the RING/U-box type E3 ligases [20,21,22]. These effectors
207 may have been acquired by bacteria via horizontal transfer from diverse eukaryotic sources
208 [42]. In addition to these types, three other classes of bacterial effectors display structures
209 completely distinct from eukaryotic E3 ligases: NELs (for Novel E3 ligase) [42], XL-box-
210 containing E3 ligases [43] and SidC ligase [44]. These ligases may represent structures evolved
211 by pathogens to mimic the functions of these essential host enzymes. The NELs family
212 encompasses effectors from different bacterial genera, including *Salmonella* (SlrP, SspH1 and

SspH2 effectors) and *Shigella* (IpaH family effectors) [20,42]. These enzymes possess an E2-interacting domain, which hijacks host E2 charged with Ubi, a cysteine residue for Ubi transfer and leucine-rich repeats (LRRs) allowing the recognition of a wide array of targets [20,42]. These different classes of E3 ligases enable bacteria to conjugate ubiquitin on specific host factors, thereby altering their stability or function, subcellular localization or interaction with other cellular proteins [20,22,42]. Bacterial E3 ligases may in particular conjugate K48-ubiquitin chains to host proteins, thereby triggering their proteasome-dependent degradation. By re-routing host factors to one of the most efficient proteolytic systems of the infected cell, bacteria manage to eliminate key host components that normally interfere with their replication and propagation [20,22]. Finally, bacterial E3 ligases can also target other bacterial effectors, co-delivered during infection, allowing a tight restriction of their activity during a specific time frame [21,22,45] (see below).

In contrast to bacterial effectors mimicking host ubiquitin enzymes, a family of proteins secreted by the bacterial pathogen *Legionella pneumophila*, the causative agent of Legionnaires' disease, was recently shown to catalyze the ubiquitination of host proteins without the need for E1 and E2 ubiquitin enzymes [46-48]. The *Legionella* SdeA effector belongs to this family of enzymes: it conjugates ubiquitin on endoplasmic reticulum (ER)-associated Rab GTPases and participates in bacterial virulence [46,47,49]. By acting independently of E1- and E2-ubiquitin enzymes, SdeA extents the repertoire of proteins potentially modified by ubiquitin. Conjugation of ubiquitin on host targets by SdeA does not rely on ATP and does not occur on lysines. SdeA instead uses NAD^+ to mediate phosphoribosyl-linked ubiquitination of serine residues in host proteins [46,47,49]. In addition to ER-associated Rab GTPases, the *Legionella* effector SdeA and other members of the Sde family ubiquitinate the host protein reticulon 4 (Rtn4), leading to ER reorganization and the formation of *Legionella*-containing vacuoles, which are intracellular factories supporting

bacteria replication [48]. Unconventional ubiquitin conjugation by Sde effectors is reversible as *L. pneumophila* codes for a specific deubiquitinase, SidJ, which removes phosphoribosylated ubiquitin from its substrate [50]. Whether functional homologs of SdeA exist in eukaryotes and what roles they may play remain to be determined.

Deconjugation of Ubiquitin and UBL from host targets by bacterial effectors

Another strategy used by bacteria to interfere with ubiquitin or UBL conjugation involves secretion into host cells of effectors with isopeptidase activity, which removes ubiquitin or UBL from their targets (Fig. 1 and Table 1). XopD, for example, is a T3SS effector secreted by the plant pathogen *Xanthomonas euvesicatoria*, which possesses SUMO-specific isopeptidase activity [51]. Upon infection of tomato cells, it deconjugates SUMO from the SIERF4 transcription factor to suppress host ethylene production, which constitutes an important pathway for plants anti-bacterial immunity [52]. Many other bacterial proteases targeting ubiquitin or UBLs have been identified in bacterial pathogens including *Salmonella*, *Shigella*, *Chlamydia*, and *Legionella*, some of them being specific for one UBL while others display cross-reactivity between different UBLs [53,54]. Interestingly, several bacterial effectors possessing a deubiquitinase activity display a strong preference for K63-linked chains over K48 or K11 chains [54]. This may reveal a significant selection pressure for bacteria to interfere with this specific ubiquitin-modification in order to promote infection.

Direct targeting of Ubiquitin and UBL polypeptides

Ubiquitin itself, as well as other UBLs, can be directly targeted and inactivated by bacterial effectors (Fig. 1). Phosphoribosylation of ubiquitin for example, catalyzed by the *Legionella* SdeA effector, was reported to interfere with multiple steps of the ubiquitination cascade [47]. The presence of phosphoribosylated ubiquitin in chains further confers resistance to various

deubiquitinases [55]. SdeA, by both triggering E1 and E2-independent ubiquitination of specific host targets and by inhibiting ubiquitination of others, thus efficiently controls the host ubiquitinome [47].

Ubiquitin and NEDD8 are also targeted by a family of bacterial T3SS effectors called Cifs (for cycle inhibiting factors), produced by diverse pathogenic bacteria such as some EPEC or *Burkholderia pseudomallei* [56]. Cifs directly target NEDD8 and ubiquitin and catalyse the deamidation of the Gln^{40} residue of these polypeptides [57]. Deamidation of ubiquitin interferes with ubiquitin chain formation, whereas deamidation of NEDD8 blocks the activity of neddylated Cullin-RING E3 ubiquitin ligases (CRLs) and impairs ubiquitination of several CRL substrates in EPEC-infected cells [57,58]. Cifs inhibit in particular the ubiquitination of Perforin-2/MPEG1 (Macrophage-expressed gene 1), an anti-microbial host protein forming pores on bacteria cells, thereby blocking its intracellular trafficking and its bactericidal activity [59].

Main host pathways targeted by bacteria and regulated by Ubiquitin or UBLs

During infection, bacterial pathogens alter the conjugation of ubiquitin or UBLs to many different host proteins. These proteins belong to different pathways that are often essential for bacteria to efficiently enter into host cells and replicate therein, or to dampen host anti-bacterial responses. We here detail some of the pathways tightly regulated by Ubi/UBL modifications and frequently targeted by bacterial pathogens.

The NF-κB pathway

The NF-κB pathway is an essential pillar of innate immunity and inflammation. Cytoplasmic NF-κB transcription factors are translocated into the nucleus within minutes after exposure to

bacteria-derived molecules and induce the transcription of a wide range of proinflammatory chemokines and cytokines [12]. Not surprisingly, many bacterial effectors target the NF-κB pathway to dampen the host innate immune response. One given pathogen may in particular produce several independent effectors targeting this pathway [12]. This apparent redundancy of effectors, that all target the same signaling cascade, reflects the diversity of danger signals sensed by the host that trigger this pathway.

One common strategy used by bacterial pathogens to dampen the NF-κB signaling cascade consists of conjugating K48-ubiquitin chains to essential components of this pathway, thereby triggering their proteasome-dependent degradation [22]. *Shigella flexneri*, for example, uses at least five different effectors that trigger ubiquitin-dependent degradation of diverse components involved in essential branches of the NF-κB pathway: IpaH1.4 and IpaH2.5 ubiquitinate the catalytic component of the linear ubiquitin chain assembly complex (LUBAC) and trigger its proteasomal degradation. Degradation of this component decreases LUBAC-mediated ubiquitination of the NF-κB modulator NEMO, which suppresses NF-κB activation [60]; NEMO may also be directly ubiquitinated by IpaH9.8 [61]; IpaH0722 ubiquitinates TRAF2, a factor involved in NF-κB pathway activation following the detection of intracytosolic bacteria [62].

Besides triggering proteasome-dependent degradation of components of the NF-κB pathway, bacterial pathogens also interfere with the endogenous ubiquitination of critical NF-κB regulators: as mentioned above, the *Shigella* OspI effector inhibits the host E2 enzyme UBC13, thereby blocking TRAF6-mediated activation of the NF-κB pathway [34]; OspG, another *Shigella* effector, binds to and inhibits the host E2 ubiquitin enzyme UBCH5, involved in IκBα ubiquitination and subsequent degradation, which is a prerequisite for NF-κB nuclear translocation and NF-κB-dependent gene transcription [63]; the NleB effector, encoded by EPEC, possesses an N-acetylglucosamine transferase activity that modifies death domains in

several proteins such as FADD and TRADD and disrupts TNF signaling including NF-κB signaling [64,65]. Interference with the NF-κB pathway may finally rely on the inhibition of ubiquitin-binding proteins, that decode ubiquitin signals into biochemical cascades [11]. TAB2 and TAB3 are two ubiquitin-chain binding proteins involved in NF-κB signaling. TAB2/3 bind K63-linked polyubiquitin chains on target proteins and activate the IκBα kinase, leading to IκBα phosphorylation, ubiquitination and degradation [66]. The NleE effector, secreted by EPEC, is an S-adenosyl-L-methionine-dependent methyltransferase that specifically modifies a zinc-finger cysteine in TAB2/3. This abolishes the binding of these proteins to ubiquitin-chains and disrupts NF-κB signaling [67].

The NF-κB pathway thereby constitutes a nice example of the diverse mechanisms evolved by bacteria to promote or inhibit ubiquitination of a large number of components in a coordinated fashion, resulting in the dampening of an essential arm of the host anti-bacterial response. Of course, these interfering strategies are not restricted to the NF-κB pathway and other important signaling cascades of the innate immune response, such as the IFN response or the activation of inflammasome, can be similarly targeted [21,68]. The *Shigella* effector IpaH9.8, for example, was reported to induce the degradation of other targets than NEMO such as GBPs (Guanylate Binding Proteins) [69,70]. Following infection, GBPs are normally recruited to bacteria-containing vacuoles or vacuole-escaped bacteria and participate to bacteria clearance. IpaH9.8 ubiquitinates GBPs with K48-linked chains and target these proteins for proteasomal degradation, thereby counteracting GBP-mediated inhibition of bacterial growth [69,70].

Autophagy

Autophagy is a cellular process by which intracellular cytosolic material is degraded by lysosomes. Specific substrates, such as intracellular pathogens, can be tagged for targeting to the autophagy pathway. They become encapsulated in *de novo*-generated double membrane

vesicles, called autophagosomes, that eventually fuse with lysosomes, leading to the degradation of their contents [71,72]. This selective autophagy is essential for cell-autonomous defense against bacteria invading the cytosol. Tagging of invading bacteria involve the deposition of a ubiquitin coat constituted by multiple polyubiquitin chains. These ubiquitin chains are synthetized by several host E3 ligases such as LRSAM1, Parkin, Smurf1, RNF166 or LUBAC [73-77]. These chains allow the recruitment of host cargo receptors that induce autophagosome formation. The host E3 ligase LUBAC generates in particular linear ubiquitin chains around intracytosolic bacteria that transform the bacterial surface into antibacterial and pro-inflammatory signaling platforms [77]. Indeed, these LUBAC-synthetized ubiquitin chains recruit host adaptors activating antibacterial immunity pathways such as the NF-κB pathway.

Professional cytosol-dwelling bacteria have evolved evasion strategies to overcome restriction by autophagy. Some of these strategies rely on interference with host ubiquitin. *Shigella flexneri*, for example, remodel ubiquitin chains normally deposited by host ligases. It antagonizes the deposition of linear ubiquitin chains by targeting LUBAC via the IpaH1.4 effector, thereby interfering with NF-κB pathway activation [60,77]. Intracellular bacteria may also evade autophagy by targeting ATG8, which regulates autophagosome biogenesis and recruitment of specific cargos during selective autophagy [78]. ATG8 actually belong to the UBL family along with SUMO, NEDD8 or ISG15. Interestingly, the RavZ effector from *L. pneumophila* was shown to target ATG8 and to inhibit autophagy [79]. This effector cleaves the amide bond between the C-terminal glycine residue and an adjacent aromatic residue in ATG8. This produces an irreversibly inactivated form of ATG8 that cannot be reconjugated [79]. This example suggests that other UBLs than SUMO, NEDD8 and ISG15 may constitute pivotal targets for pathogens to promote infection.

Host cytoskeleton

Remodeling of the host cytoskeleton is frequently used by intracellular bacterial pathogens to enter into the targeted cells, create a niche where they can efficiently replicate, and disseminate to neighboring cells [80]. Several components of the host cytoskeleton are regulated by ubiquitin. RhoGTPases, for example, which control actin cytoskeleton dynamics, are degraded by the proteasome following ubiquitin conjugation [81]. Interestingly, the ubiquitination level of RhoGTPases can be modulated during *Salmonella* infection, suggesting that this bacterium may modulate RhoGTPase turn-over [82]. SUMO can be conjugated to different components of the host cytoskeleton as well, including actin itself and actin regulatory proteins, septins or intermediate filaments such as keratins and lamins [83,84]. The role of ubiquitin and UBL modifications in the regulation of the cytoskeleton is only in its infancy but one can anticipate that it may represent an important target for bacterial pathogens to manipulate the cell architecture.

Transcription factors

In order to exploit host functions, bacterial pathogens remodel the proteome of infected cells. This remodeling may result from deregulation of gene transcription by injection of bacterial proteins such as nucleomodulins that act directly on the host nucleus [85], or by interference with host transcription factors, some of them being regulated by ubiquitin or UBLs. *L. monocytogenes*, for example, dampens the SUMOylation of numerous transcription factors during infection [36]. As SUMO conjugation either increases or decreases transcription factors activity, this decrease in SUMOylation may modulate the expression of a specific subset of genes and lead to a reprogramming of host gene expression. As mentioned above, decreasing the SUMOylation of host transcription factors is a strategy also used by the plant pathogen *Xanthomonas euvesicatoria* that specifically targets SUMO-SIERF4 to dampen the host ethylene-mediated antibacterial response [52]. Finally, the colibactin toxin, produced by some

388 *Escherichia coli* strains in the intestine, induces a downregulation of the SUMO isopeptidase
389 SENP1 and an increase in the SUMOylation of the transcription factor p53. This ultimately
390 results in the emergence of senescent cells secreting growth factors that may promote colorectal
391 carcinogenesis [86].
392
393 *PML Nuclear Bodies*
394 PML (Promyelocytic Leukemia Protein) is a protein that polymerizes in discrete nuclear
395 assemblies known as PML nuclear bodies (NBs) and plays essential roles in many different
396 cellular processes [87]. Key to its function, PML can be post-translationally modified by SUMO
397 [87]. In addition to its role in anti-viral host defense [18], PML was recently identified as a
398 sensor for bacteria producing pore-forming toxins [37]. Indeed, intoxication of human cells by
399 the Listeriolysin O toxin, secreted by *L. monocytogenes*, triggers a massive deSUMOylation of
400 PML. This deSUMOylation of PML, coupled to an oxidative stress-dependent multimerization
401 of PML, initiates host cell anti-bacterial responses leading to a decrease in *Listeria* intracellular
402 replication [37]. This example of PML highlights how SUMO alterations of some specific host
403 proteins can constitute danger signals for the cells that triggers back anti-bacterial responses.
404 The putative role of PML in other bacterial infections targeting host SUMOylation, such as
405 *Shigella* or *Salmonella*, remains unknown but deserves further investigation.
406
407 **Post-translational modifications of bacterial proteins during infection**
408 Besides interfering with host protein post-translational modifications, bacteria can hijack host
409 ubiquitin or UBL-conjugation machineries to modify their own components (Fig. 1). As for
410 eukaryotic proteins, conjugation of ubiquitin or UBL has diverse effects on bacterial effectors
411 and may change their intracellular localization, their stability or their interaction with other
412 bacterial or host factors [88]. Post-translational modification of bacterial proteins couples their

activity to their arrival into the host cell cytoplasm. Interestingly, post-translational modification of bacterial proteins can also be used by the host to tag exogenous proteins and target them for degradation [88].

Ubiquitination of *Salmonella* proteins constitute nice examples illustrating the versatility of consequences of this post-translational modification on bacterial proteins activity. SopE and SptP are two *Salmonella* effectors that contribute to the transient remodeling of the host cell's cytoskeleton [89]. These two effectors, which are delivered simultaneously by *Salmonella*, exhibit different half-lives. SopE, which is involved in actin cytoskeleton rearrangement, membrane ruffling and bacteria uptake, is rapidly polyubiquitinated and degraded by the host proteasome [89]. SptP, which deactivates the RhoGTPases turned on by SopE, exhibits much slower degradation kinetics, allowing recovery of the actin cytoskeleton's normal architecture a few hours after infection [89]. Conjugation of ubiquitin to SopB, a phosphoinositode phosphatase secreted by *Salmonella* via T3SS, modifies its cellular localization [90]. Upon delivery, SopB associates with the host plasma membrane where it participates in actin-mediated bacterial entry. Later on, ubiquitination of SopB by TRAF6 leads to its translocation to *Salmonella*-containing vacuoles, where it modulates vesicle trafficking and interferes with the delivery of these vacuoles to lysosomes [90,91]. Mass spectrometry-based large-scale analysis of the ubiquitinome of cells infected by *Salmonella* recently provided additional examples of bacterial proteins modified by ubiquitin [82]. In addition to SopE and SopB, several effectors were identified as being ubiquitinated during infection [82]. Interestingly, integral outer membrane proteins were reported to be conjugated to ubiquitin and may represent the targets forming the ubiquitin coat that surrounds intracytosolic bacteria and is involved in host anti-bacterial autophagy [71,82].

In contrast to ubiquitination, only a few bacterial proteins have been reported to be modified by SUMO and the biological consequences of these modifications during infection often remains

438 elusive [92,93]. These SUMO-modified bacterial proteins include two effectors, TRP120 and
439 AmpA, secreted by two intracellular pathogens, *Ehrlichia chaffeensis* and *Anaplasma*
440 *phagocytophilum* respectively [92,93]. OspF, an effector secreted by *Shigella flexneri*,
441 constitutes another example for which SUMO conjugation is required for the translocation of
442 this effector into the host nucleus where it modulates the expression of proinflammatory
443 cytokines [94].
444 One can anticipate that recently developed techniques for large scale proteomic studies of UBL
445 conjugation will increase the list of bacterial proteins modified by SUMO or other UBLs, and
446 provide new insights for the role of these modifications during infection.
447

448 **Concluding Remarks**

449 Ubiquitin and UBL are essential post-translational modifiers of eukaryotic cells. Thousands of
450 Ubi/UBL targets have been identified during recent years, suggesting that most proteins will be
451 modified by this type of PTM at some point in their cellular lifetime. It is thus not surprising
452 that pathogens have evolved so many strategies to interfere with these particular PTMs in order
453 to manipulate host cell physiology. Interfering with host Ubi/UBL modifications is observed
454 both for intracellular pathogens, that tightly interact with host cell cytoplasmic components to
455 create for example a protective niche where they can acquire nutrients from the host, and for
456 extracellular pathogens, that manipulate host cells to favor their maintenance at the surface of
457 the cells or dampen host immune responses.
458 Two types of interfering strategies used by bacterial pathogens can be distinguished: they may
459 either globally dampen Ubi/UBL systems, by targeting the conjugation machineries or by
460 modifying the equilibrium between conjugation/deconjugation reactions, or they may alter the
461 level of Ubi/UBL-conjugation of only specific host proteins involved in bacterial proliferation
462 and antibacterial responses. In the case of global dampening of Ubi/UBL systems, a wide range

463 of host proteins display altered levels of Ubi/UBL conjugation. Discrimination, in this pool of
464 proteins, of host factors directly involved in infection from other factors that only represent "co-
465 lateral damages" may constitute a real challenge to study the exact role of these PTMs during
466 infection.
467 Thanks to the continuous improvement in proteomic analyses, the list of proteins known to be
468 modified by ubiquitin or UBLs has greatly expanded during these last years. For example, it is
469 now feasible to compare the variations of the ubiquitinome (or other "UBL-ome") of cells
470 during infection by a pathogen or after exposure to a bacterial toxin [36,82]. Some of these
471 techniques are furthermore compatible with *in vivo* analysis and the comparison of the content
472 of proteins modified by Ubi/UBL in organs from infected or control animals is now possible
473 [95,96]. Interestingly, current proteomic-based approaches have not only revealed the identity
474 of the proteins modified by Ubi/UBL but also the modification sites. These data are critical for
475 further analysis of the role of these PTMs in the function of the identified protein and hence, to
476 decipher the consequences of bacterial alteration of these PTMs. Several recent studies on
477 ubiquitin conjugation have revealed that ubiquitination establishes a much more complex code
478 than originally thought. Indeed, in addition to "mixed" ubiquitin chains involving different
479 types of linkages between ubiquitin monomers, chains that mix ubiquitin with other UBLs such
480 as SUMO have also been reported [4,27,97]. In addition, ubiquitin has recently been found to
481 be itself post-translationally modified by acetylation or phosphorylation, which further expands
482 the repertoire of ubiquitination [4,27,97]. We are only beginning to understand the tremendous
483 diversity of ubiquitin modifications and their roles in cell biology but it is very likely that
484 bacterial pathogens have long learned how to break this so-called "ubiquitin code" and
485 efficiently use it for their own profit (see Outstanding Questions).
486 Finally, while this review has focused on pathogenic bacteria, some non-pathogenic
487 bacteria such as commensals of the intestinal microbiota were also reported to interfere with

488 host Ubi/UBL systems [98]. For example, production of butyrate by commensal bacteria leads
489 to the inactivation of the E2 NEDD8 enzyme in intestinal epithelial cells and was proposed to
490 participate in the inflammatory tolerance of gut bacteria [99,100].

491 Manipulation of Ubi/UBL conjugation by pathogenic bacteria constitute a key facet of
492 host-pathogen interactions. Studying how bacteria interfere with these PTM is essential to
493 complete our understanding of the infection process. In particular, identification of bacterial
494 effectors harboring non-eukaryotic enzymatic activities and manipulating host Ubi/UBL may
495 provide potential new drug targets, which is critical in this age of bacterial resistance to
496 antibiotics.

Figure 1, Key Figure : Main strategies used by bacterial pathogens to interfere with host ubiquitin or ubiquitin-like protein modifications.

During infection, bacteria may inhibit or trigger the degradation of Ubi/UBL enzymes such as E1 activating enzymes, E2 conjugating enzymes or E3 ligases (1). These effects can be directly triggered by bacterial effectors, or indirectly via the induction of host signaling cascades (dashed lines). Bacteria may also secrete effectors possessing either E3 ligase activity, which conjugate Ubi/UBL to host targets (2), or isopeptidase activity, which remove Ubi/UBL from their targets (3). Bacteria may also directly modify ubiquitin or UBLs to block their conjugation to host targets (4). Finally, bacteria may hijack the host Ubi/UBL systems to modify their own proteins during infection (5). Red arrows, proteins secreted by bacteria.

507 **Table 1 : Examples of bacterial proteins interfering with Ubi/UBL conjugation to host proteins**

508

Ubi/UBL target	Bacteria	Extra/intracellular bacteria	Effector	Enzymatic activity	Effect	Refs
Ubiquitin	*Salmonella* Typhimurium	intracellular	SopA	E3 Ubi ligase (HECT)	Regulation of host inflammation	[67]
Ubiquitin	EPEC, EHEC	extracellular	NleL	E3 Ubi ligase (HECT)	Regulation of actin pedestal formation	[101]
Ubiquitin	EPEC, EHEC	extracellular	NleG	E3 Ubi ligase (RING)	?	[102]
Ubiquitin	*Pseudomonas syringae*	extracellular	AvrPtoB	E3 Ubi ligase (U-box)	Inhibition of plant pattern-triggered immunity	[103,104]
Ubiquitin	*Shigella flexneri*	intracellular	OspI	Gln deamidase	Inactivation of UBE2N/UBC13 (E2 Ubi enzyme NF-κB pathway)	[34]
Ubiquitin	*Shigella flexneri*	intracellular	IpaH1.4	E3 Ubi ligase (NEL)	Ubiquitination of LUBAC (NF-κB pathway)	[60]
Ubiquitin	*Shigella flexneri*	intracellular	IpaH2.5	E3 Ubi ligase (NEL)	Ubiquitination of LUBAC (NF-κB pathway)	[60]
Ubiquitin	*Shigella flexneri*	intracellular	IpaH0722	E3 Ubi ligase (NEL)	Ubiquitination of TRAF2 (NF-κB pathway)	[62]
Ubiquitin	*Shigella flexneri*	intracellular	IpaH9.8	E3 Ubi ligase (NEL)	Ubiquitination of NEMO (NF-κB pathway)	[61]
Ubiquitin	*Salmonella* Typhimurium	intracellular	Ssph1	E3 Ubi ligase (NEL)	Ubiquitination of PKN1 (NF-κB pathway)	[105]
Ubiquitin	*Legionella pneumophila*	intracellular	SdeA	non eukaryotic Ubi ligase	E1/E2-independent ubiquitination of Rab GTPases and RTN4	[46-48]
Ubiquitin	*Shigella flexneri*	intracellular	OspG	kinase	Inhibition of UBCH5 (E2 Ubi enzyme; NF-κB pathway)	[63]
Ubiquitin	EPEC, EHEC	extracellular	NleB	Glycosyltransferase	Inhibition of TRAF2 ubiquitination (NF-κB pathway)	[106]
Ubiquitin	EPEC	extracellular	?	?	Downregulation of UBE1 and UBA6 (E1 Ubi enzymes)	[35]

Ubiquitin	EPEC	extracellular	NleE	Cys methyltransferase	Inactivation of TAB2 and TAB3 (NF-κB pathway)	[67,107]
Ubiquitin	*Legionella pneumophila*	intracellular	SidJ	deubiquitylase	?	[50]
Ubiquitin	*Shigella flexneri*	intracellular	ShiCE	deubiquitylase	?	[54]
Ubiquitin	*Chlamydia trachomatis*	intracellular	ChlaDUB1	deubiquitylase	Inhibition of NF-κB pathway activation	[108,109]
Ubiquitin	*Burkholderia pseudomallei*	extracellular	CHBP	Gln deamidase	Deamidation of Ubiquitin	[57]
SUMO	*Listeria monocytogenes*	intracellular	LLO	Pore-forming toxin	Downregulation of UBE2I/UBC9 (E2 SUMO enzyme	[25]
SUMO	*Clostridium perfringens*	extracellular	PFO	Pore-forming toxin	Downregulation of UBE2I/UBC9 (E2 SUMO enzyme	[25]
SUMO	*Streptococcus pneumoniae*	extracellular	PLY	Pore-forming toxin	Downregulation of UBE2I/UBC9 (E2 SUMO enzyme	[25]
SUMO	*Shigella flexneri*	intracellular	? / Ca^{2+} influx	?	Proteolytic cleavage of UBA2/SAE2 (E1 SUMO enzyme)	[39]
SUMO	*Salmonella* Typhimurium	intracellular	? / miRNAs	?	Downregulation of UBE2I/UBC9 (E2 SUMO enzyme	[38]
SUMO	*Xanthomonas euvesicatoria*	extracellular	XopD	deSUMOylase	DeSUMOylation of SIERF4 (plant immune response)	[51,52]
NEDD8	EPEC	extracellular	CIF	Gln deamidase	Deamidation of NEDD8	[57,58]
NEDD8	*Chlamydia trachomatis*	intracellular	ChlaDUB1	deNeddylase	Inhibition of NF-κB pathway activation	[108,109]

EPEC, Enteropathogenic *Escherichia coli*. EHEC, Enterohaemorragic *Escherichia coli*. HECT, homologous to the E6-AP carboxyl terminus. RING, really interesting new genes. NEL, Novel E3 ligase. ?, not determined.

Text Box 1

Even if bacteria do not encode Ubiquitin, two types of bacterial polypeptides that can be linked to target proteins via isopeptide bonds have been reported [110,111]. The first type of bacterial modifier is called Pup (for prokaryotic ubiquitin-like protein) and is restricted to specific bacterial phyla such *Actinobacteria*. Pupylation, *i.e.* the covalent fixation of Pup to target proteins is a fascinating example of convergent evolution with respect to eukaryotic ubiquitination. It involves an enzymatic machinery distinct from ubiquitination, that deamidates Pup C-terminal Gln before linking it to target lysine residues [110,111]. This covalent modification targets bacterial proteins to proteasomal degradation. Several hundred of pupylated targets have been identified to date, which are involved in a variety of pathways. In *Mycobacterium tuberculosis*, pupylation is essential for the survival of bacteria in the host and may thus constitute an interesting drug target,[110-112]. The second type of bacterial modifiers is constituted by polypeptides that differ from Ubi in sequence but share a common structural ß-grasp fold [110]. These Ubi-fold proteins include the archeal SAMPs (small archeal modifier proteins) and *Thermus* TtuB (tRNA-two-thiouridine B) that function both as protein modifiers and sulfur carriers for sulfur-containing biomolecules synthesis [110]. Bacterial Ubi-fold proteins linkage to lysine residues in target proteins involves ubiquitin E1 homologs but not canonical E2 or E3 ubiquitin-like enzymes [110].

In addition to these modifiers analogous to ubiquitin, some human commensal bacteria may even usurp eukaryotic ubiquitin for their own purpose. Indeed, an ubiquitin gene has been identified in the genome of *Bacteroides fragilis* strains [113,114]. Interestingly, this eukaryotic-like ubiquitin, which was probably acquired via horizontal gene transfer, does not seem to be involved in bacterial protein modification since it lacks the critical terminal glycine residue. This protein is instead secreted and acts as a bacterial toxin targeting and killing other intestinal bacteria [114]. Many other surprises like this one are probably still awaiting to be discovered

and, even though the first report of a bacterium post-translationally modifying a host protein occurred almost 50 years ago [115], the field of pathogen and host post-translational modifications is, without a doubt, still very promising.

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