Hijacking the Host Ubiquitin Pathway: Structural Strategies of Bacterial E3 Ubiquitin Ligases

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Running Title: Bacterial E3 Ubiquitin Ligases

Summary

Ubiquitinylation of proteins is a critical mechanism in regulating numerous eukaryotic cellular processes including cell cycle progression, inflammatory response, and vesicular trafficking. Given the importance of ubiquitinylation, it is not surprising that several pathogenic bacteria have developed strategies to exploit various stages of the ubiquitin pathway for their own benefit. One such strategy is the delivery of bacterial 'effector' proteins into the host cell cytosol, which mimic the activities of components of the host ubiquitin pathway. Recent studies have highlighted a number of bacterial effectors that functionally mimic the activity of eukaryotic E3 ubiquitin ligases, including a novel structural class of bacterial E3 ligases that provides a striking example of convergent evolution.

Introduction

Several pathogenic bacteria utilize specialized type III or type IV secretion systems to deliver bacterial proteins, called effectors, into host cells to modulate a variety of cellular pathways. There are a growing number of effectors that usurp the host ubiquitin pathway by functionally mimicking components of the pathway. Ubiquitinylation results in the covalent attachment of ubiquitin to a lysine residue on a target protein [1]. Following the initial conjugation, subsequent ubiquitin molecules can be ligated to one of seven lysines in the previously attached ubiquitin molecule, resulting in polyubiquitinylation of various linkages. Therefore, a substrate can be monoubiquitinylated at a single lysine residue, multi-ubiquitinylated at multiple lysine residues, or polyubiquitinylated at one or more lysine residues. The type of ubiquitinylation and the topology of the ubiquitin chains formed direct substrate fate [2]. Ubiquitinylation can signal for proteasome-dependent degradation or function as non-proteolytic signals important for DNA repair, signal transduction and vesicular trafficking [3–7].

Ubiquitinylation involves an enzymatic cascade resulting in the formation of an isopeptide bond between ubiquitin and internal lysine residues of a substrate protein [8]. This process involves an ubiquitin-activating enzyme (E1), which forms a thioester bond between a catalytic cysteine and the carboxy terminal glycine residue of ubiquitin. The ubiquitin is then transferred to an ubiquitin-conjugating enzyme (E2). Finally, an ubiquitin ligase (E3) facilitates the covalent conjugation of ubiquitin from an ubiquitin-loaded E2 to one or more lysine residues in the substrate. Therefore, E3 ubiquitin ligases confer specificity to the reaction through substrate binding. E3 ubiquitin ligases are defined by their ability to facilitate the transfer of

ubiquitin from a cognate E2 to a specific substrate. There are two major known types of E3 ubiquitin ligases in eukaryotes, which possess distinct structural and mechanistic properties: the RING (really interesting new gene)/U-box domain and the HECT (homologous to E6-associated protein C terminus) domain [9,10]. In this review, we focus on the structures features used by bacterial effectors to mimic the activity of eukaryotic E3 ubiquitin ligases.

RING/U-box-like E3 Ubiquitin Ligases

Eukaryotic RING/U-box E3s mainly function as scaffolds to facilitate the transfer of ubiquitin directly from an E2 to a substrate. RING domains are defined by the consensus sequence $Cx_2Cx_{9-39}Cx_{1-3}Hx_{2-3}C/Hx_2Cx_{4-48}Cx_2C$, which forms a 'cross-brace' motif [11]. This motif coordinates the binding of two zinc ions between alternating Cys and His residues. In the U-box variant, the Cys and His residues are replaced with charged and polar residues that mediate salt bridges and hydrogen bonds resulting in a similar overall structure [12]. RING/U-box domains also include a concave surface consisting of a three amino acid hydrophobic patch that binds to a conserved sequence present on most E2s [11,13]. RING domains are often part of a multidomain protein or a multisubunit complex. While the RING/U-box domain recruits ubiquitin-loaded E2s, an additional domain or subunit that encodes a protein-protein interaction motif, such as scr homology-2 or leucine-rich repeat (LRR) domain, is required for substrate recognition.

The type IV effector LubX of *Legionella pheumophila* has two domains both with striking sequence similarity to eukaryotic E3 U-box domains. U-box1 of LubX was shown to have ligase activity, while U-box2, which lacks a hydrophobic residue critical for interaction with E2s, is inactive [14]. When the three-dimensional structure of the U-box1 of LubX is modeled using the PHYRE threading program, the structure and positioning of key residues is very similar to known RING/U-box proteins [15] (Fig. 1 A and B). As predicted, mutations disrupting the putative E2 binding domain of U-box1 abolished ubiquitin ligase activity of LubX. Interestingly, U-box2 functions to bind host Cdc2-like kinase 1 (Clk1) and targets it for ubiquitinylation by U-box1 *in vitro*. Therefore, *Legionella* likely modulates Clk1 function during infection by translocating LubX, a RING-like E3 ubiquitin ligase. The consequence of Clk1 ubiquitinylation during *Legionella* infection remains to be determined.

However unlike LubX, most known bacterial E3 ligases do not share sequence similarity with eukaryotic E3s. The *Pseudomonas syringae* effector AvrPtoB is an example of a structural mimic of the RING/U-box family of E3 ligases. *Pseudomonas syringae* pathovar *tomato* causes bacterial speck disease on tomato and *Arabidopsis* [16]. In susceptible plants, *Pseudomonas syringae* injects the type III effector AvrPtoB, which suppresses programmed cell death (PCD) [17]. Structural studies revealed that the C-terminal domain of AvrPtoB adopts a 'core fold' nearly identical to the RING/U-box domain of the human E3, Rbx1, consisting of a three-stranded sheet with a single helix and two extended loops (Figure 1A). Similar to LubX, AvrPtoB also encodes a highly-conserved binding site for host E2s, where the positioning of critical residues is preserved (Figure 1B) [18,19]. Experiments confirmed that AvrPtoB possesses ubiquitin ligase activity and this activity is required to suppress PCD during *Pseudomonas syringae* infection. Therefore, while undetectable at the sequence level, the potential biochemical activity of AvrPtoB was revealed by its structural homology to RING/U-box E3 ligases. Subsequent studies have shown that the N-terminal domain of AvrPtoB mediates the recruitment of substrates and directs the ubiquitin ligase activity of the C-terminal domain. The N-terminal domain of AvrPtoB binds to several host kinases important in plant immunity, such as tomato Fen kinase, Flagellin-sensing receptor kinase 2, and the Chitin elicitor receptor kinase 1, resulting in their ubiquitinylation and proteosomal degradation [20–22]. Therefore, AvrPtoB promotes *Pseudomonas syringae* virulence by targeting signaling proteins important for plant immunity for ubiquitin-mediated degradation.

HECT-like E3 Ubiquitin Ligases

HECT-type E3 ubiquitin ligases are modular proteins consisting of an N-terminal substrate binding domain and a HECT domain characterized by a ~350 residue C-terminal domain with a conserved catalytic cysteine residue ~35 residues from the C terminus [23]. Unlike RING/U-box E3 ubiquitin ligases, HECT E3s participates directly in the catalysis of ubiquitinylation by forming a covalent thioester intermediate between the catalytic Cys of the HECT domains and the C-terminus of ubiquitin prior to transferring ubiquitin to the target protein. The HECT domain consists of two subdomains: the N-terminal lobe (N lobe), which binds E2s, and the C-terminal lobe (C lobe), which possesses the catalytic Cys. The N and C lobes are connected by a flexible loop, and while the overall structure of HECT domains is similar, the orientation and position of the lobes can vary [24]. The structural organization of most HECT domains requires that a substantial conformational change occurs to bring the catalytic Cys of the HECT domain close enough to the E2 active site for ubiquitin transfer [25,26]. Mutations that restrict the rotation of the N and C lobes about the flexible loop reduce ubiquitin ligase activity, supporting the requirement for such a conformational change for catalytic activity [25]. This conformational flexibility is thought to enable the movement of ubiquitin from the E2 to the catalytic cysteine of the HECT E3 and then finally to the substrate.

The *Salmonella enterica* type III effector protein SopA structurally mimics HECT E3 ubiquitin ligases despite lacking significant amino acid similarity. SopA regulates host inflammatory responses, which leads to the transepithelial migration of polymorphonuclear (PMN) cells during *Salmonella* infection [27]. *Salmonella* strains expressing an E3 ligase-defective mutant of SopA induced less PMN transepithelial migration indicating that the E3 ligase activity of SopA is important for SopA function [27].

SopA is organized into an N-terminal putative substrate binding domain and a C-terminal HECT-like domain (Figure 2A). Similar to the human HECT E3 ligase E6-AP, the HECT domain of SopA is comprised of N and C lobes that are orientated in a L-shape [28]. The N lobe of SopA forms the long arm while the C lobe, which contains a catalytic Cys located 30 residues from the C-terminus, forms the short arm of the L structure. The lobes of SopA are connected by a flexible linker helix, which likely allows for the conformational changes required for ubiquitin transfer.

While there is minimal sequence similarity around the active site of SopA in comparison to other HECT E3 ligases, alignment of the active site loop sequence revealed that Leu747 and Thr752 in addition to catalytic Cys753 of SopA are conserved in all known HECT domains [28]. Alanine replacement of either of these residues reduced ubiquitin ligase activity.

The N-terminal putative substrate-binding domain of SopA forms a parallel -helix structure, similar to those found in the pectin lyase-like structural superfamily, which is involved in carbohydrate binding [28]. While the presence of a β-helix motif suggests that SopA may ubiquitinylate carbohydrate-modified substrates, the substrates for SopA area still unknown. Interestingly, the substrate-binding domain of SopA extends from the opposite end of the N lobe structure of SopA as compared to the structure of the HECT-type E3 ligase E6-AP (Figure 2B). Yet Diao et al. showed that placement of the β-helix domain near the putative E2 binding region neither obstructed nor contributed to E2 binding of SopA [28]. The placement of the putative substrate-binding domain of SopA is markedly different from that observed in eukaryotic HECT E3s and will require additional co-structures of SopA with its cognate E2 and/or substrates are needed to help characterize the conformational changes required for ubiquitin transfer from SopA to its substrate (Figure 2B).

NEL E3 Ubiquitin Ligases

Recently, a new family of E3 ubiquitin ligases has been described that possesses a structural domain (termed NEL for <u>N</u>ovel <u>E</u>3 <u>L</u>igase), which is distinct from either the RING or HECT domains. NEL E3 ligases comprise a large family of a bacterial effector proteins encoded by a subset of pathogenic bacteria (Table 1). Prior to being recognized as ubiquitin ligases, NEL E3 ligases were classified as leucine-rich repeat (LRR) effector proteins due to the presence of an N-terminal LRR domain. In 2007, Rohdes et al. discovered that two family members, *Shigella* spp. IpaH9.8, and *Salmonella*, SspH1, possessed E3 ubiquitin ligases activity despite lacking sequence similarity to any known E3 ubiquitin ligases [29]. Since uncovering the ubiquitin ligase activity of this family of effector proteins, three structural studies have shed light on this new class of E3 ubiquitin ligases.

The nearly full-length structures of two NEL E3 ubiquitin ligases, *Salmonella* SspH2 and *Shigella* IpaH3, reveal similar folds composed of two well-defined structural elements: a N-terminal LRR domain linked by a short stretch of residues to a novel C-terminal helical domain with ubiquitin ligase activity (Figure 3A) [30,31]. Both LRR domains resemble a curving solenoid made of either 12 (SspH2) or 9 (IpaH3) LRR motif repeats capped at the N- and C-terminal regions with α-helices. The inner, concave surfaces of both LRR domains are lined with hydrophobic residues consistent with their putative involvement in protein-protein interactions. LRR domains provide a versatile platform for protein-protein interactions whereby simply varying the number of repeats, the curvature of the structure or the surface characteristics can alter binding specificity.

The C-terminal NEL domain forms a unique fold that possesses ubiquitin ligase activity. It consists of 2 subdomains: an N-terminal globular region followed by two long finger-like helices extending from the globular fold [30–32]. The E3 ubiquitin ligase activity of the NEL domain requires a single conserved Cys residue. Similar to HECT E3s, this conserved Cys can act as a nucleophile forming a thioester bond with ubiquitin [31,32]. IpaH9.8 of *Shigella* contains several charged residues close to the catalytic Cys that are required for ligase activity, including Asp339, Arg340 and Asp387 [32]. These residues, as well as their positioning, are conserved in all NEL E3 effectors and likely participate directly in the ubiquitinylation reaction and/or substrate binding.

While the structures of both SspH2 and IpaH2 are composed of very similar N-terminal LRR domain and C-terminal NEL domains, there is a dramatic difference in the orientation of these domains with respect to each other (Figure 3). In SspH2, the concave surface of the LRR curves towards the NEL domain, partially occluding the putative protein-binding surface of the LRR domain and burying the catalytic Cys residue. In contrast, the LRR domain of IpaH3 is fully extended resulting in a solvent accessible catalytic Cys residue. We hypothesize that the structure of SspH2 depicts an autoinhibited or closed conformation, while the structure of IpaH3 represents an activated conformer in which the binding surface of the LRR is available and the catalytic cysteine accessible. A transition from the close conformation to the active conformation would result from a change in orientation of the NEL domain and the LRR domain translated through the linker region between the two domains (Figure 3B). We propose a model in which activation results in a relative reorientation of the two domains that exposes the catalytic site to the host ubiquitinylation machinery and substrate(s). Consistent with this model, mutations that destabilize the binding interface between the LRR and NEL domains of SspH2 dramatically increase the ubiquitin ligase activity of full-length SspH2 [30].

The role of NEL E3 ligase dependent ubiquitinylation during infection remains elusive. While overexpression of IpaH9.8 leads to the degradation of the mitogen-activated protein kinase (MAPK) kinase Ste7 in yeast, no decrease in components of the mammalian MAPK pathway was observed following *Shigella* infection [29]. In addition, *Salmonella* SspH1 and SlrP have

been shown to target PKN1 and thioreodoxin respectively *in vitro*, but *in vivo* ubiquitinylation of either target has yet to be demostrated [29,33]. Therefore, *in vivo* targets of NEL E3 ligases during infection remain to be identified.

Conclusion

Several pathogenic bacteria have evolved effectors that have the capacity to exploit the host ubiquitin pathway by functionally mimicking eukaryotic E3 ubiquitin ligases (Table 1). Some bacteria accomplish this by expressing effectors, such as *Legionella* LubX, with significant amino acid sequence similarity to eukaryotic E3 ligases, which may have been acquired through horizontal gene transfer from a eukaryotic host. However, many effectors successfully mimic the activities of E3 ligases despite lacking amino acid sequence similarity. For example, *Pseudomonas* AvrPtoB and *Salmonella* SopA structurally mimic known eukaryotic E3 ligases by adopting homologous structures. While others, belonging to the NEL E3 ligase family, have evolved a novel structure to functionally mimic eukaryotic E3 ligases activity.

The discovery of the E3 ligase activity for these effectors is an exciting first step towards understanding the functional role they play during infection. However, much remains to be learned about these fascinating molecules. What are the types of ubiquitin conjugates that are formed by these different ligases? What are the host enzymes (i.e. E2s) they engage to carry out their function? And finally and arguably most important to understand their actual role in infection, what are the substrates they target? Understanding the mechanisms by which these enzymes restrict their activity to specific cellular locations would also help to clarify their role in host-pathogen interactions. In addition to broadening our understanding of bacterial pathogenesis, the study of bacterial E3 ubiquitin ligases will likely provide insights into the basic biology of the host ubiquitinylation pathway and may lead to the development of novel therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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• of special interest

•• of outstanding interest

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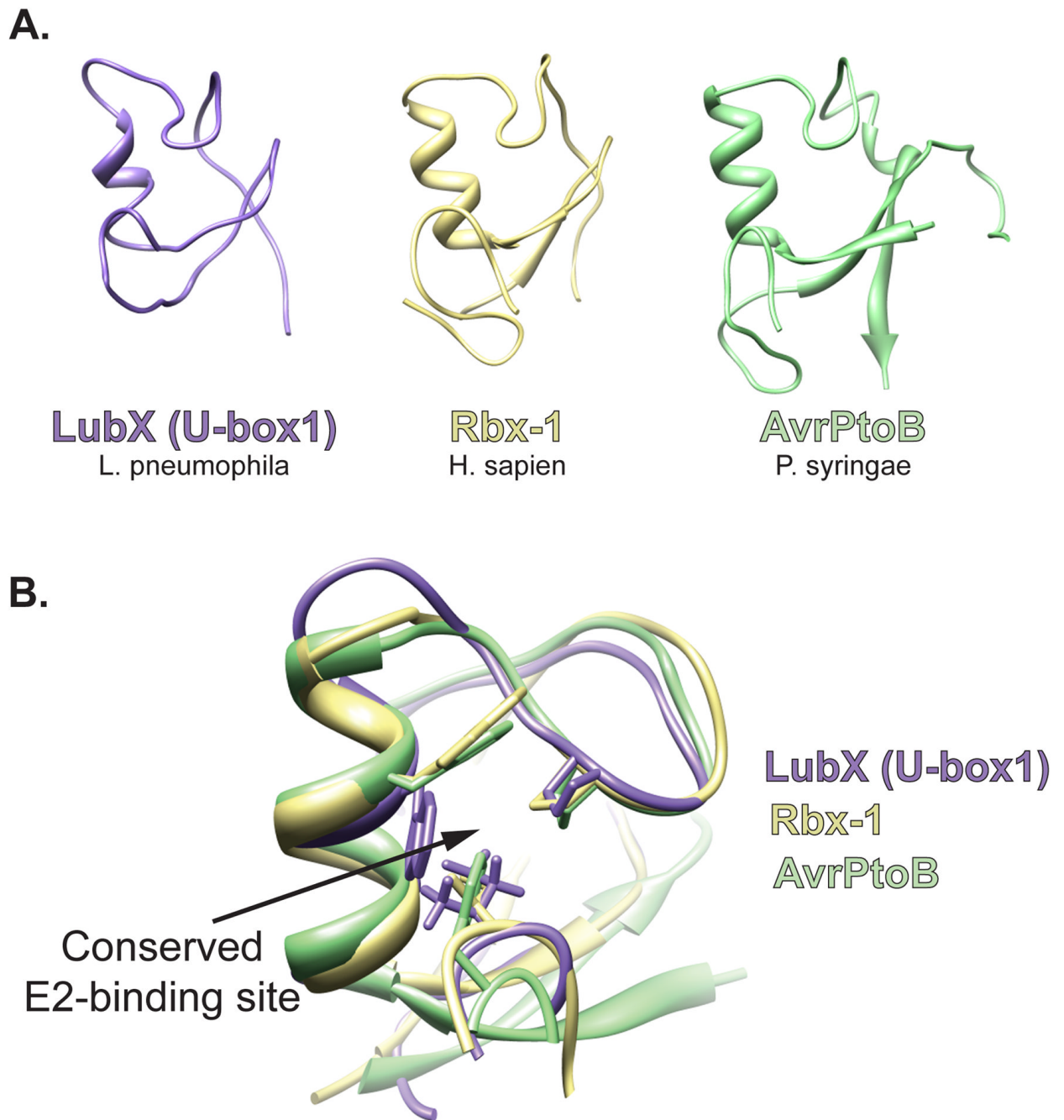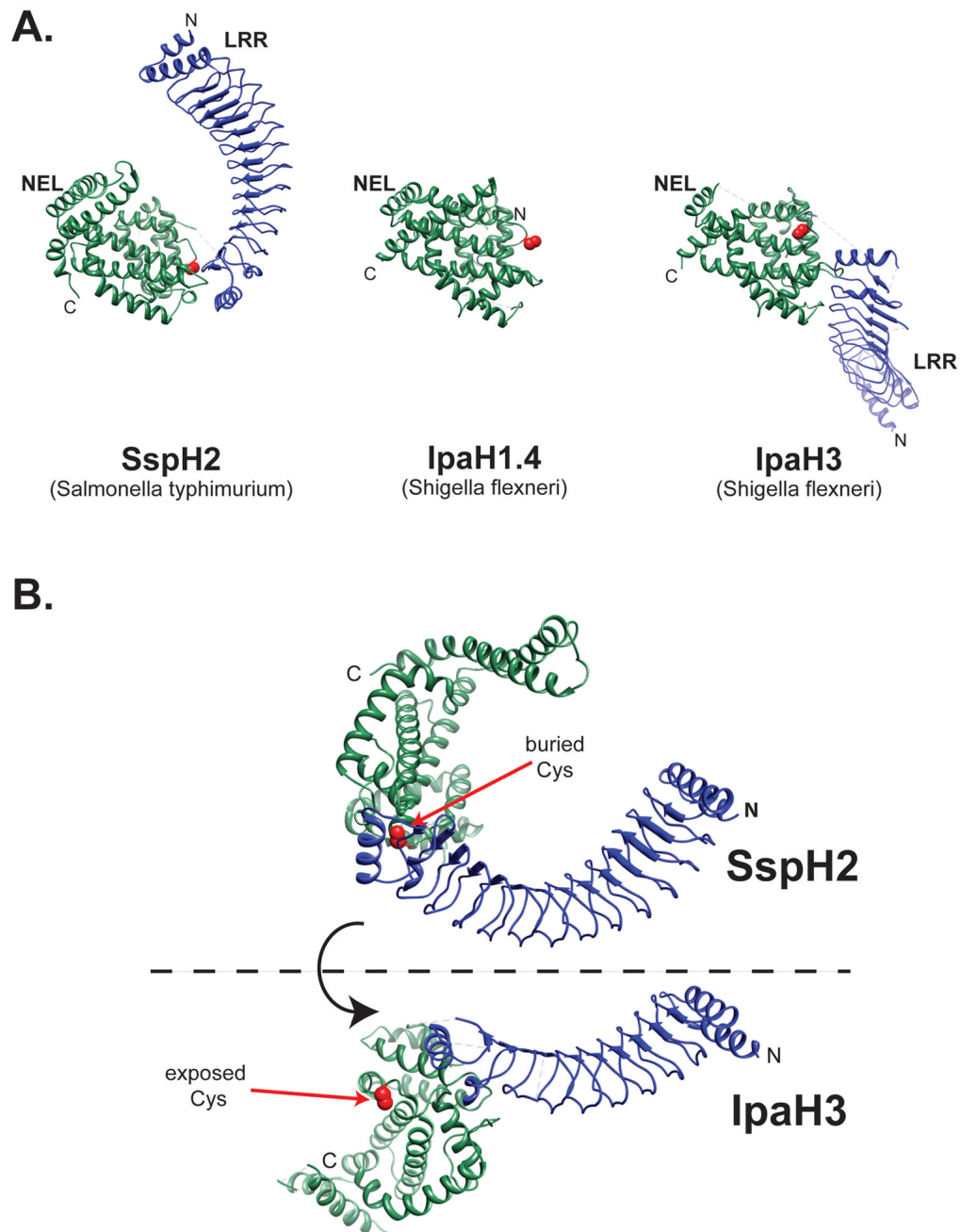
Figure 1.
Bacterial mimics of eukaryotic RING/U-box E3 ligases. **(A)**. Using the Phyre threading program, the sequence of U-box1 of *L. pneumophila* LubX was aligned to known structures and the structure was modeled to its best fit, human E3 traf6 (E-value of $2.6e^{-11}$; estimated precision of 100%); the RING/U-box structure of H. sapien, Rbx-1 (PDB ID 3DPL); the core fold of P. syringae, AvrPtoB (PDB ID 2FD4). **(B)** Visualization of the E2-binding site residues of Rbx-1 with homologous regions in LubX and AvrPtoB. The three putative E2-binding residues are shown.

A.

SopA

B.

HECT E3   **SopA**
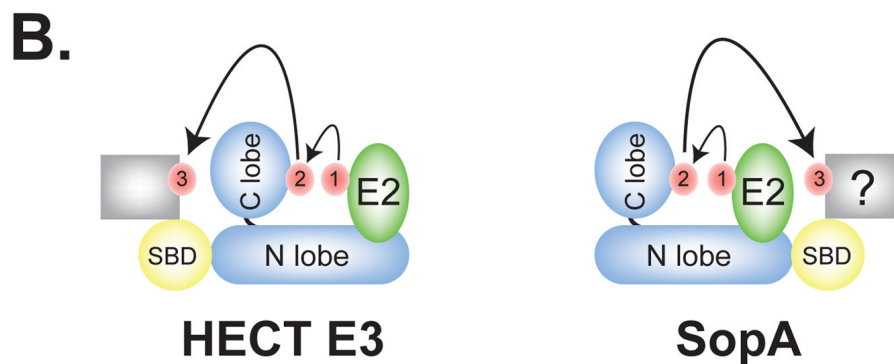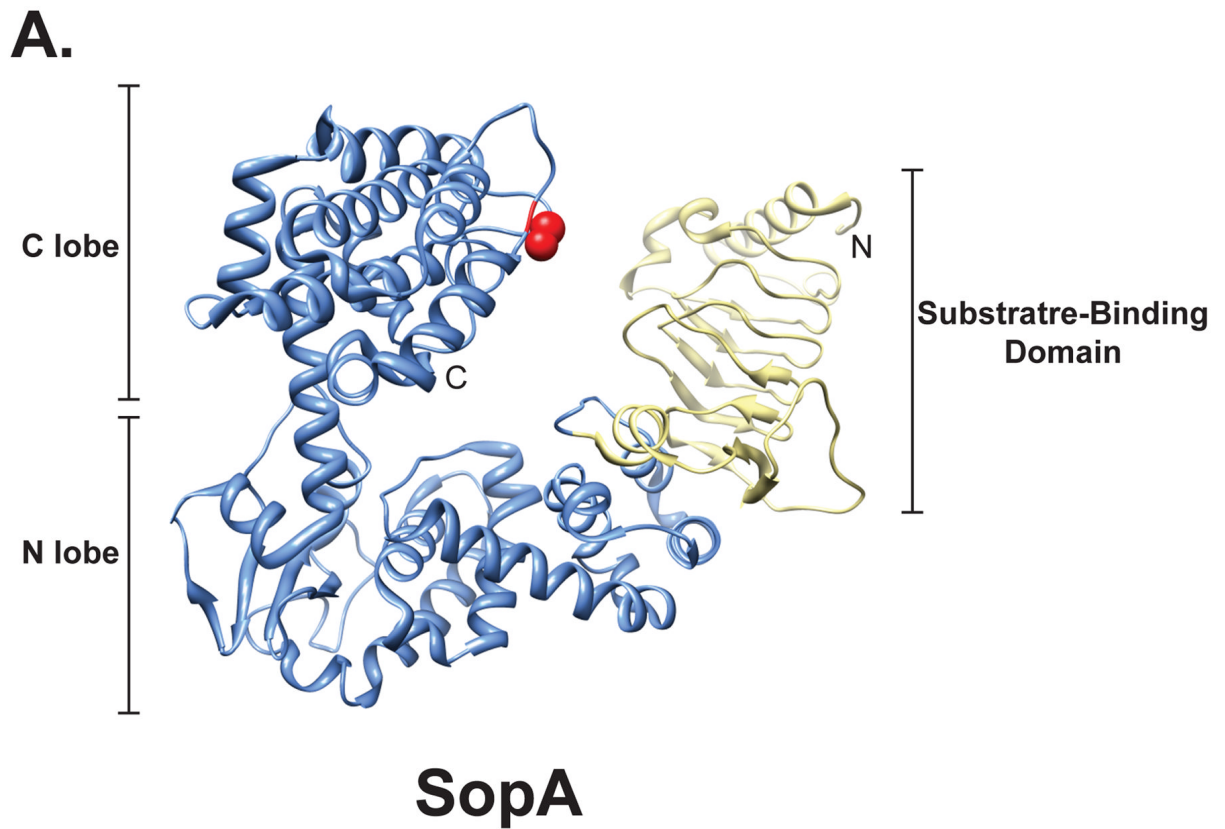
Figure 2.
SopA is a HECT-like E3 ligase. **(A)** Overall structure of SopA$_{163-782}$ (PDB ID 2QYU); the HECT domain of SopA is shown in blue and the N-terminal β-helix domain in yellow. The catalytic cysteine is signified in red. N, NH2 terminus; C, COOH terminus. **(B)** Schematic diagram of the transfer of ubiquitin. Ubiquitin is shown in red. The C lobe of a generic HECT E3 or SopA is positioned similarly to accept ubiquitin following binding of Ub-charged E2 (green). However, the placement of the substrate binding (SBD; yellow) domain of SopA on the opposite end of the N lobe as found in eukaryotic HECT E3s likely requires a significantly different conformational change to facilitate ubiquitin transfer to a target substrate (grey box).

Figure 3.
NEL family of E3 ligases. **(A)** The structure of SspH2$_{166-783}$ (PBD ID 3G06),
IpaH1.4$_{265-575}$ (PDB ID 3CKD), and Ipa3$_{25-561}$ (PDB ID 3CVR) is shown with leucine-rich
repeat (LRR) domain in blue and the novel E3 ligase (NEL) domain in green. The catalytic
cysteine residue is shown in red. N, NH2 terminus; C, COOH terminus. **(B)** Conformational
changes likely activate the NEL family of E3 ligases. The structures of *Shigella* IpaH3 and
Salmonella SspH2 suggests a dramatic hinge motion as the NEL domain rotates 180° from the
closed conformation indicated by the structure of SspH2 to the open position represented by
the IpaH3 structure. The catalytic cysteine residue is shown in red. N, NH2 terminus; C, COOH
terminus.

Table 1

Examples of Bacterial E3 Ligase Mimics

Bacteria	Effector	E3 Structural Family	Host Targets	Refs
Bradyrhizobium spp	Blr1676	NEL	Unknown	[31]
	Blr1904	NEL	Unknown	[31,32]
Escherichia coli ssp	Ecol5_01000486	NEL	Unknown	[34]
	Ecol5_01001536	NEL	Unknown	[34]
	Ecol5_01001967		Unknown	[34]
	Ecol5_01003958	NEL	Unknown	[34]
	Ecol5_01004202	NEL	Unknown	[34]
	Ecol5_01004539	NEL	Unknown	[34]
	Ecol5_01004764	NEL	Unknown	[34]
	Ecol5_01004830	NEL	Unknown	[34]
	Ecol5_01004885	NEL	Unknown	[34]
	NleG2–3	RING/U-box	Unknown	[35]
Legionella spp	LubX	RING/U-box	Clk1?	[15]
Pseudomonas spp	AvrPtoB	RING/U-box	Fen, CERK1, FSL2	[18–22]
	PflO1_4099	NEL	Unknown	[31]
	PflO1_4565	NEL	Unknown	[31]
	PP_2212	NEL	Unknown	[32]
	PP_2394	NEL	Unknown	[32]
	PSPTO_1492	NEL	Unknown	[31]
	PSPTO_4093	NEL	Unknown	[31]
Rhizobium spp	Y4fR	NEL	Unknown	[31,32]
Salmonella ssp	Slrp	NEL	Thioredoxin?	[30,33]
	SopA	HECT-like	Unknown	[27,28]
	SspH1	NEL	PKN-1?	[29,30]
	SspH2	NEL	Unknown	[30]
Shigella ssp	IpaH1	NEL	Unknown	[34]
	IpaH1.4	NEL	Unknown	[32]
	IpaH2	NEL	Unknown	[34]
	IpaH2.5	NEL	Unknown	[34]
	IpaH3	NEL	Unknown	[31]
	IpaH4	NEL	Unknown	[34]
	IpaH4.5	NEL	Unknown	[31]
	IpaH5	NEL	Unknown	[34]
	IpaH6	NEL	Unknown	[34]

Bacteria	Effector	E3 Structural Family	Host Targets	Refs
	IpaH7	NEL	Unknown	[34]
	IpaH7.8	NEL	Unknown	[31]
	IpaH9.8	NEL	STE7?	[32]
Yersinia spp	YPA_3361 [1]	NEL	Unknown	[31,34]
	YPA_3364 [1]	NEL	Unknown	[31,34]

[1] these are representative open reading frames from *Yersinia pestis* Angola that are found in most *Yersinia pestis* and *Yersinia pseudotuberculosis* strains