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Function, molecular mechanisms, and therapeutic potential of bacterial HtrA proteins: An evolving view

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ABSTRACT

Members of the high temperature requirement A (HtrA) protein family are widely distributed amongst prokaryotic and eukaryotic species. HtrA proteins have ATP-independent dual chaperone-protease activity and mediate protein quality control. Emerging evidence indicates that HtrA family members are vital for establishing infections and bacterial survival under stress conditions. Bacterial HtrA proteins are increasingly thought of as important new targets for antibacterial drug development. Recent literature suggests that HtrA protein AlgW from *Pseudomonas aeruginosa* has distinct structural, functional, and regulatory characteristics. The novel dual-signal activation mechanism seen in AlgW is required to modulate stress and drug responses in bacteria, prompting us to review our understanding of the many HtrA proteins found in microorganisms. Here, we describe the distribution of HtrA gene orthologues in pathogenic bacteria, discuss their structure–function relationships, outline the molecular mechanisms exhibited by different bacterial HtrA proteins in bacteria under selective pressure, and review the significance of recently developed small molecule inhibitors targeting HtrA in pathogenic bacteria.

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1. Introduction

Highly conserved HtrA (high-temperature requirement A) proteins, also known as Deg (degradation of periplasmic proteins), were first identified in *Escherichia coli* in 1989 [1]. They are envelope-associated heat shock serine proteases found in organisms ranging from prokaryotes to humans [2,3]. The chaperone and proteolytic functions of HtrAs are required for the folding and maturation of secreted protein. HtrA family members consist of a serine-protease domain and at least one C-terminal regulatory PDZ domain [3,4]. All HtrA proteins can assemble into oligomers,

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which range in size from trimers to hexamers to 12-mers and even 24-mers [5,6]. HtrA proteins usually reside in a catalytically inactive state, which can be converted into a reversible activated state by substrate allostery or by non-substrate peptide binding mediated by the PDZ domain [4,7,8].

Eukaryotic HtrA proteins, such as human HtrA1-3, have key roles in many cellular physiological and pathological processes, including cancer, neurodegeneration, arthritis, age-related macular degeneration, and familial ischemic cerebral small-vessel disease [9,10]. In prokaryotes, HtrA family members are crucial to bacterial survival and adaptation to environmental change [11–15]. Pathogenic bacteria use HtrA proteins to overcome external stresses. The loss of HtrA function correlates with reduced virulence and restricted bacterial growth under stress. Evidence indicates that HtrA family members are responsible for alginate production, biofilm formation, morphological transformation, and can confer antibiotic resistance [16,17]. Certain HtrA proteins can be secreted from the cell to facilitate bacterial invasion [18]. Their significant physiological functions, particularly those in virulence and antibiotic resistance, suggests that members of the HtrA family constitute attractive drug targets [10,19–22].

Previous reviews of the HtrA proteins have surveyed their biochemical properties, localization, functions in specific species, and their roles in different disease states [2,9,10,23,24]. Given the wealth of recent studies delineating new activation mechanisms and virulence functions of HtrA proteins in pathogens [16,25], we will seek to evaluate how the composition, diversity, and assembly processes of bacterial HtrAs impinge on their roles in bacterial resistance and pathogenesis, highlighting the increased interest in HtrA inhibitors as novel antimicrobial agents.

2. Distribution and evolution of HtrA family proteins in pathogenic bacteria

E. coli DegS, DegP, and DegQ proteins are the best studied members of the HtrA family [26]. All of these proteins are essential for

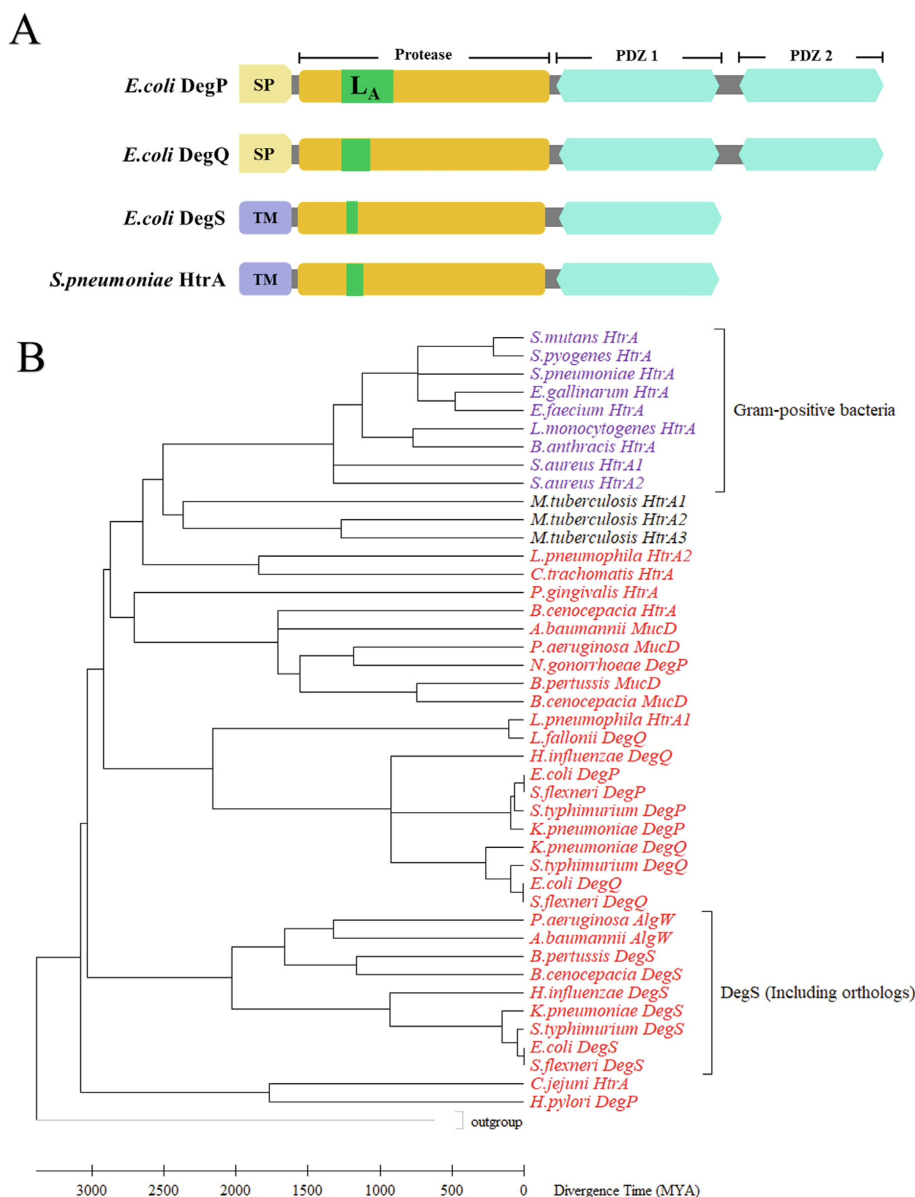

Fig. 1. Domain assembly and gene distribution of HtrA proteins in pathogenic bacteria. (A) Schematic domain diagram of HtrA proteins from pathogenic bacteria. The protease domain is colored orange, the PDZ domain is colored cyan, and L_A is colored green. SP stands for signal sequence. TM stands for transmembrane region. (B) A phylogenetic tree of HtrA proteins with an approximate divergence time. Gram-positive bacteria are colored purple and Gram-negative red. MYA: million years ago.

protein quality control in the *E.coli* periplasmic space [2]. They represent the two major structural subdivisions within the family: DegP/Q and DegS. DegP/Q proteins contain one serine-protease domain and two PDZ domains, and their N-terminal signal peptides are able to mediate cytoplasmic membrane translocation [23]. DegS proteins have a single C-terminal PDZ domain. Their N-terminal transmembrane segments are able to mediate inner-membrane anchoring (Fig. 1A).

DegP/Q and DegS homologues are evident in most well-studied Gram-negative pathogenic bacteria [10], including *Burkholderia cenocepacia, Pseudomonas aeruginosa, Klebsiella pneumoniae, Legionella pneumophila, Porphyromonas gingivalis,* and *Salmonella Typhimurium*. In *Mycobacterium tuberculosis* and Gram-positive pathogens, including *Listeria monocytogenes, Staphylococcus aureus,* and *Streptococcus pneumonia*, only membrane anchored HtrAs are present, which are similar to DegS (Fig. 1A).

Despite low overall sequence identity, HtrA proteins can be identified relatively using the PDZscape database [27]. Making use of HtrA protein sequences from 25 representative pathogenic bacteria, we undertook a phylogenetic analysis and Ancestor Sequence Reconstruction (ASR) [28]. As expected, DegP/Q-like HtrAs cluster together (Fig. 1B). The resulting phylogenetic tree indicates that DegS-like HtrAs are present in Gram-negative and Gram-positive bacteria, implying that HtrAs existed before the divergence of Gram-negative and Gram-positive bacteria. It also suggests HtrAs may have additional functions that are more complex in Gram-negative bacteria, since such bacteria have greater periplasmic complexity, and that HtrA evolutionary information may have been lost in Gram-positive bacteria.

Part of the conserved protease domain, the functional LoopA (L_A), has distinctly different lengths and sequences in the DegP/Q group and the DegS groups (Fig. 1A), suggesting this feature may be useful when classifying DegP, DegQ, and DegS sequences and structures [3,6,29,30]. Since the L_A loop is an essential regulatory element of HtrA proteins [16,31], the sequence and structural diversity of this region may be useful when exploring HtrAs function and evaluating structure–function relationships within the DegP/Q and DegS subgroups.

3. The versatile roles exhibited by HtrA proteins in bacteria

The main functions of HtrAs are thought to be regulating stress responses and controlling protein quality in Gram-negative periplasm [32,33]. Recent work suggests HtrA proteins are also responsible for many other vital processes, particularly infection (Fig. 2) [16,34]. *E. coli* DegP was originally identified as a serine protease capable of cleaving misfolded proteins at Val-X or Ile-X peptide motifs at 37–45 °C, with protease activity enhanced after membrane interaction [11,12]. Later studies indicated that DegP could switch between chaperone and ATP-independent protease activities in a temperature-dependent manner, with both activities crucial to rescuing the cell from stress-induced lethality [35].

The role of DegP in virulence has also been observed in many bacteria. *E. coli* DegP is needed for the efficient assembly of

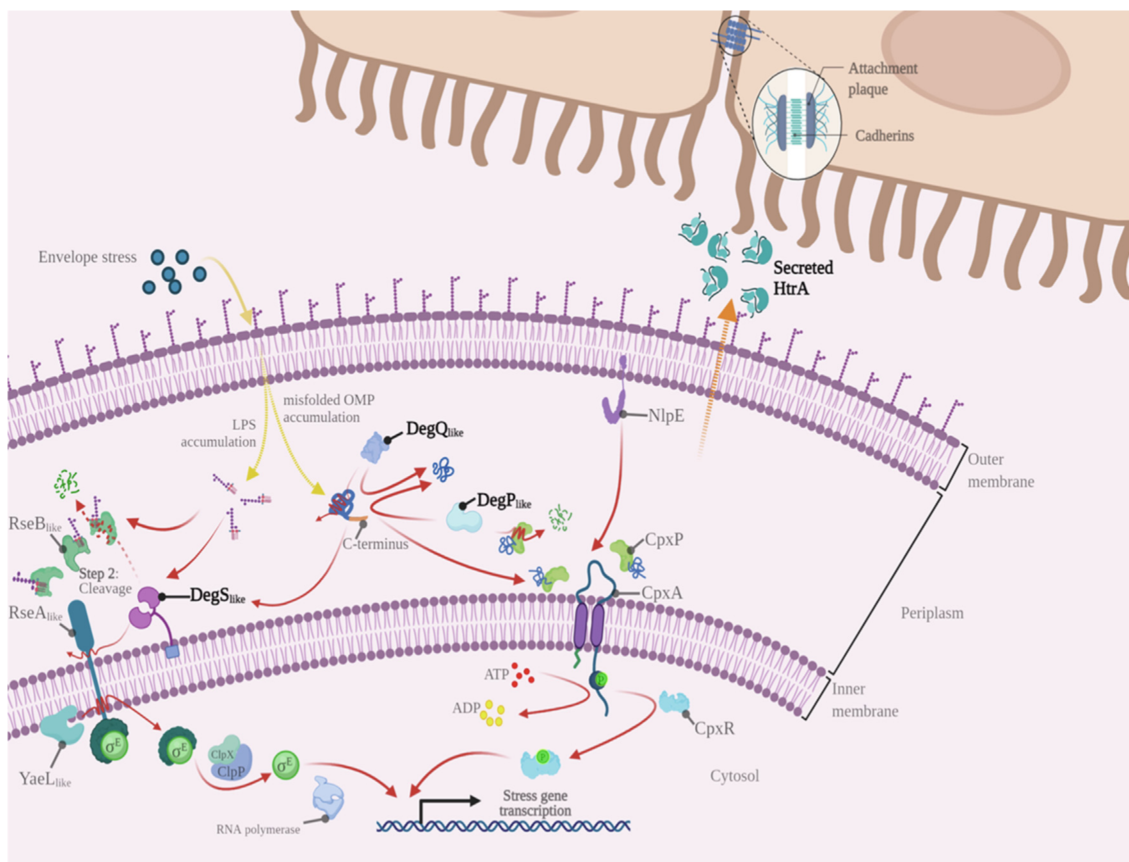

Fig. 2. Schematic diagram of bacterial HtrA functions. In the RIP pathway, misfolded proteins activate DegS-like protein and/or disrupt the RseB-like:RseA-like complex. DegS-like and YaeL-like proteins, together with the ClpXP complex, process the RseA-like protein initiating stress-response gene expression. In the periplasm, DegQ-like and DegP-like proteins both have chaperone activity under normal conditions. DegP-like protein also has protease activity, degrading CpxP-misfolded protein complexes and activating CpxR-mediated transcription under stress conditions. In some pathogenic bacteria, secreted HtrA protein typically participates in bacterial colonization and host invasion. LPS: lipopolysaccharide; OMP: outer membrane protein.

bundle-forming pili (BFP). BFP is critical for host-cell attachment [36]. Mutants of *S. flexneri* DegP could impair intercellular spread, probably due to the role of DegP in regulating IcsA surface presentation [37]. Recently, DegP has been shown to correlate with acid resistance required for food-borne enteric pathogens to pass through the human stomach [38]. *P. aeruginosa* MucD is believed to be involved in biofilm formation and also influences host neutrophil responses [39–41].

Prior work has demonstrated that DegP is regulated by the CpxAR two-component regulatory system consisting of CpxA, CpxR, and CpxP [42]. The CpxRA pathway induces bacterial responses by sensing extra-cytoplasmic stress, such as altered pH, the presence of copper ions, or the over production of envelope protein, leading to enhanced transcription of stress genes, such as HtrA [43]. CpxR acts as a response regulator and CpxA acts as a sensor kinase, which is suppressed by the CpxP dimer [44]. When lipoprotein transport is defective or periplasmic protein folding is overloaded, the CpxP/CpxA complex dissociates as the misfolded proteins bind to the interface of CpxP. The outer-membrane anchored lipoprotein NlpE then interacts with the periplasmic domain of CpxA to trigger its kinase activity [45]. CpxA autophosphates His151, shifting this phosphate group to Asp51 of CpxR. Subsequently, phosphorylated CpxR acts as a transcription factor, facilitating specific gene transcription [46]. Complexes of CpxP with misfolded protein are finally degraded by DegP [47].

When compared to DegP, DegQ has similar substrate specificity, moderate sequence identity, and a shorter loop L_A [48]. DegQ was initially identified as a substitute for DegP in protein quality control. It was able to rescue the temperature sensitive phenotype, although viability was not affected significantly in DegQ mutants [49]. DegQ is not essential for cell survival at high temperatures. This indicates DegQ mainly, but not completely, compensates for the absence of DegP under heat stress [49]. Several studies show that DegQ is associated with pH variation and has higher chaperone activity than DegP. Low pH induces remodeling of the DegQ particle. The null *degQ* mutant affected the growth of *E. coli* in a low pH environment. Taken together these observations indicate that DegQ may play an important role in acid resistance rather than temperature sensitivity [50]. However, a detailed physiological role for DegQ remains elusive [14].

DegS was also identified as a DegP homologue [49]. However, DegS could not completely rescue temperature-sensitive phenotypes of DegP mutants. The loss of DegS results in a small-colony phenotype [49]. Later, DegS was shown to act as an envelope stress sensor able to cleave the periplasmic segment of RseA, which activates RpoE (known as σ^E) [51]. The Regulated Intramembrane Proteolysis or RIP pathway is a well-characterized regulatory network in bacteria, activated by misfolded envelop proteins, which transduces stress signals from the environment (Fig. 2) [51]. Under normal conditions, another periplasmic protein (RseB) acts as a negative regulator of σ^E activation by binding RseA to prevent DegS degradation [52,53]. Oxidative stress, heat shock, or secretion stress leads to the periplasmic aggregation of misfolded proteins. The interaction of RseB and RseA is disrupted by free LPS. This causes the C-terminal domain of RseA to be exposed, allowing DegS cleavage and the activation of σ^E. The transcription factor σ^E then interacts with RNA polymerase to promote transcription of several regulators, including HtrA, DegP, RpoE, and RpoH [54]. Moreover, the ResB homologue MucB could sense lipid molecules and be degraded by DegS homologue AlgW in *P. aeruginosa*; something which has yet to be observed in *E. coli* [52].

DegS is also activated allosterically by the C-terminal YxF sequence of misfolded outer membrane proteins (OMPs), initiating a proteolytic cascade through the cleavage of the periplasmic domain of RseA, allowing the subsequent activation of σ^E [7]. Recent work indicates *P. aeruginosa* DegS homologue AlgW is a novel HtrA protease activated by both peptide and lipid signals [16]. HtrA proteins may thus directly sense off-pathway LPS and OMPs, transmitting outer-membrane stress signals to downstream pathways.

In Gram-positive bacteria, HtrAs have also been shown to participate in virulence regulation and biofilm formation [12,55]. Recently, HtrA inactivation has been shown to facilitate the resistance of Gram-positive pathogens such as *S. aureus* and *S. pneumoniae* to antibiotics that target cell walls [17,56]. Whereas in *M. tuberculosis*, HtrA (RV1223) can block the toxicity of a cell wall amidase and thus maintain homeostasis [57]. In the Gram-positive spore-producing bacterium *Bacillus anthracis*, strains with *htrA* gene deletions exhibited increased sensitivity to stress, downmodulation of the bacterial S-layer, and attenuated virulence [58].

HtrA proteins can be translocated to the cell surface or into the extracellular environment, where secreted HtrAs often participate in bacterial colonization or host invasion [26,59]. The mechanism underlying extracellular HtrA localization has been proposed to consist of (a) anchoring at the bacterial surface; (b) secretion into the extracellular matrix; and (c) transport through the OMV [26].

Direct HtrA involvement in bacteria-host interaction has been observed experimentally in both Gram-positive and Gram-negative bacteria. In *B. anthracis*, HtrA is anchored at the bacterial surface and is probably secreted into the extracellular matrix after auto cleavage [60]. HtrA proteins have also been detected in *H. influenzae*, *H. pylori*, *C. jejuni*, and *A. actinomycetemcomitans* [10]. A recent study indicates that HtrA proteins in *H. pylori* are related to the risk of gastric cancer and a subsequent study suggested a time-specific host–microbe relationship probably exists in some gastrointestinal pathogens [61,62]. In Gram-negative bacteria, OMVs seem to be an effective means of translocation for virulence-associated HtrAs. However, the detailed mechanism of HtrA release remains unclear [26].

Bacterial pathogens can penetrate the epithelial barrier via tight junction proteins targeted by extracellular HtrA [34,63,64]. E-cadherin is an essential cell adhesion protein and tumor suppressor in host epithelia tissues. It is able to mediate cell–cell contact and regulate the permeability of the epithelial barrier [65]. E-cadherin degradation by HtrA facilitates colonization and pathogen transmigration during infection. This conjecture has been verified in certain gastrointestinal bacteria, including *H. pylori* and *C. jejuni* [10]. Recent studies indicate that the PDZ1 domain rather than the PDZ2 domain of *H. pylori* HtrA is necessary for full cleavage activity and growth under stress conditions. Truncated *H. pylori* HtrA proteins retain protease activity and can damage E-cadherin-based adherens junctions [66]. Claudin-8 and occludin are also substrates for HtrA proteins [63,64]. Since similar epithelial cells exist in the lung, pneumonia pathogens using HtrA-mediated E-cadherin cleavage to penetrate the blood-air barrier should be investigated. Extracellular HtrA proteins should thus be targeted by antimicrobial drugs in order to treat bacterial infections and help combat bacterial drug resistance.

4. Structure characteristics and activity relationships of HtrA proteins

The structure of HtrA proteins comprises a funnel-like homotrimer, consisting mainly of protease domains. The protease domain of all HtrA proteins is structurally conserved. It belongs to the S1C protease fold, part of the SA clan [24]. It comprises two β-barrels flanked by α-helices (Fig. 3A and 3B). The Ser–His–Asp catalytic triad lies in a crevice between the two β-barrels and is surrounded by activation loops L_1-L_3, L_D, and L_A [13]. It is known that L_A plays important roles in HtrA function, including sensing temperature change, mediating higher oligomerization, and regulating substrate access to the active site [31].

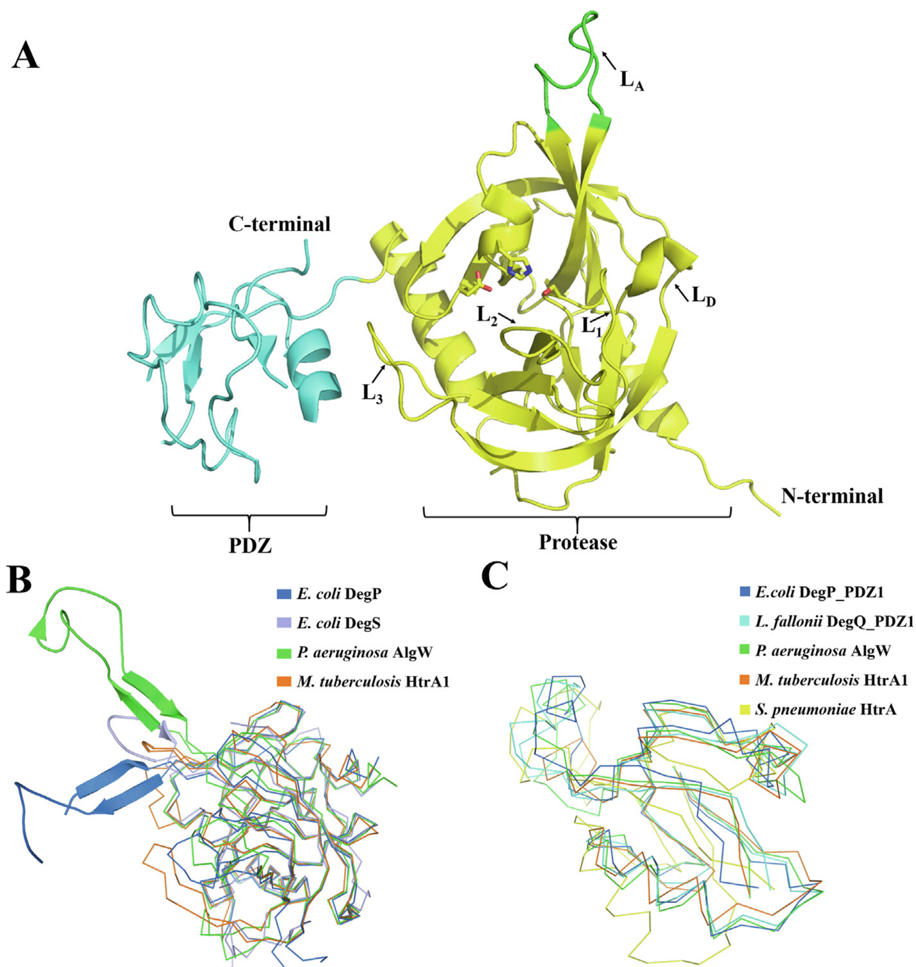

Fig. 3. Structure characteristic of HtrA. (A) Overall structure of *E. coli* DegS monomer (1SOZ) [5]. DegS consists of a PDZ domain (cyan) and a protease domain (yellow). The catalytic triad (His96, Asp126, Ser201), surrounded by activation loops L_1, L2, L3, LD, and LA, is shown using yellow sticks. (B) Superposed protease domains from *E. coli* DegP (blue), *E. coli* DegS (periwinkle blue), *P. aeruginosa* AlgW (green), and *M. tuberculosis* HtrA1 (orange). L_A is rendered using a protein cartoon. (C) Superposed PDZ domains from *E. coli* DegP (blue), *L. fallonii* in DegQ (cyan), *P. aeruginosa* AlgW (green), *M. tuberculosis* HtrA1 (orange), and *S. pneumoniae* HtrA (yellow).

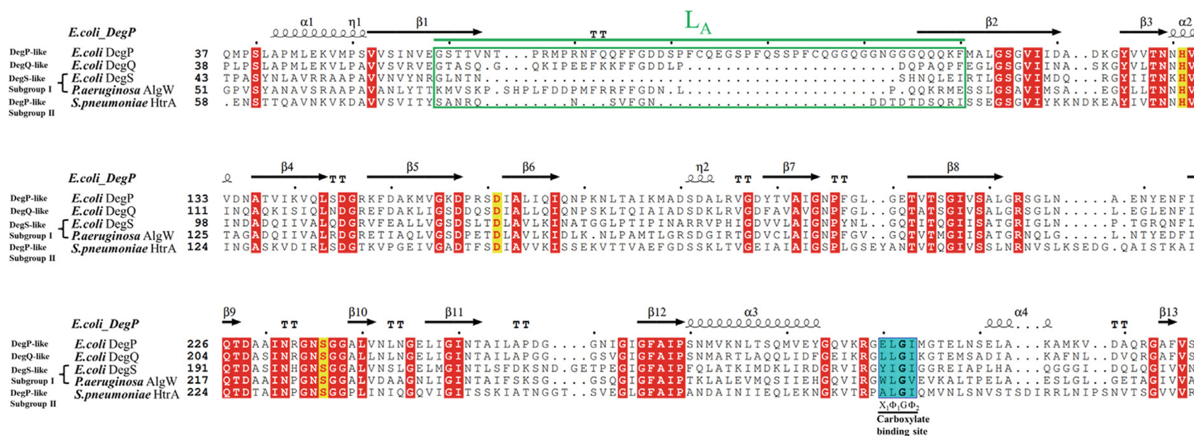

Fig. 4. Sequence alignment of HtrA protease domain. 43 HtrAs from 25 representative pathogens divide into four groups: DegP-like, DegQ-like, and DegS-like Subgroup I and II. Considering the space constraints, we selected a representative sequence from each group for next sequence alignment. The experimental secondary structure of *E. coli* HtrA is included at the top of the sequence. Loop L_A is shown in a green box, the carboxylate-binding loop (X_1-Φ_1-G-Φ_2) in a blue box with a cyan background, and the catalytic triad (His, Asp, Ser) is colored red on a yellow background.

L_A loops from different HtrA subgroups have different lengths. As shown in Fig. 4, L_A loops from the DegP/Q group are much longer than the corresponding loops from DegS proteins. The DegS group can be further subdivided into L_A-long (represented by *P. aeruginosa* AlgW), L_A-short (represented by *E. coli* DegS), and L_A-medium (represented by Gram-positive *S. pneumoniae* HtrA).

Considering that the flexibility of *P. aeruginosa* AlgW L_A is tightly associated with activation processes [16], extended L_A segments in L_A-long and L_A-medium DegS-like HtrA proteins may have extra functions.

Unlike the conserved protease domain structure, HtrA PDZ domains exhibit greater sequence variation and higher conformational plasticity [33]. The PDZ domain, a widespread protein module of 80–100 amino acids, regulates protein–protein interactions and mediates the assembly of protein complexes that initiate signal transduction [67]. Comparison of PDZ domains from HtrA proteins indicates overall structure similarity, but also local structural differences (Fig. 3C). In the *S. pneumonia* HtrA PDZ domain, five β-strands form a β-sandwich flanked by two α-helices. This is similar to the canonical PDZ fold and belongs to Class II PDZ domains. The PDZ1 domain from *E. coli* DegP comprises two anti-parallel β-strands and two α-helices. Earlier reports concluded that the hydrophobic pocket or "$X_1\Phi_2G\Phi_2$" loop in the HtrA PDZ domain specifically recognizes and accommodates the C-terminus of target peptides (Fig. 4). The additional PDZ2 domain in DegP/Q alters the domain orientation of PDZ1 and maintains protein integrity, and is also critical for the assembly of HtrA proteins into higher oligomeric states [68].

5. Activity regulation of HtrA proteins

HtrA has only limited activity in its resting state. Several factors including pH, temperature, substrate binding, or allosteric effectors, such as peptides from outer membrane proteins, are required for activation [8]. It has been shown that binding to substrate proteins is necessary for efficient conversion of inactive trimeric or hexameric *E. coli* DegP to large active cage-like oligomers. However, cage assembly and proteolytic activation can be uncoupled, and the allosteric activation of inactive trimers into proteolytically active forms by substrate binding is an intrinsic property of DegP [69]. Structures of $DegP_6$ indicate that L_A loops extend outwards, forming β-strand interactions with L_A–L_A corner pillars. Loop L_A also interacts with L_1 and L_2 to block the reaction center. Reduced L_A–L_A interactions could lead to $DegP_6$ disassembly and the formation of $DegP_{12}$ or $DegP_{24}$, resulting in exposed active catalytic triads (Fig. 5A and 5B). Zarzecka *et al.* observed the conversion of higher molecular weight oligomeric forms of *C. jejuni* HtrA into lower molecular weight isoforms after full cleavage of the substrate, which confirms the dynamic interconversion of diverse oligomeric forms [25].

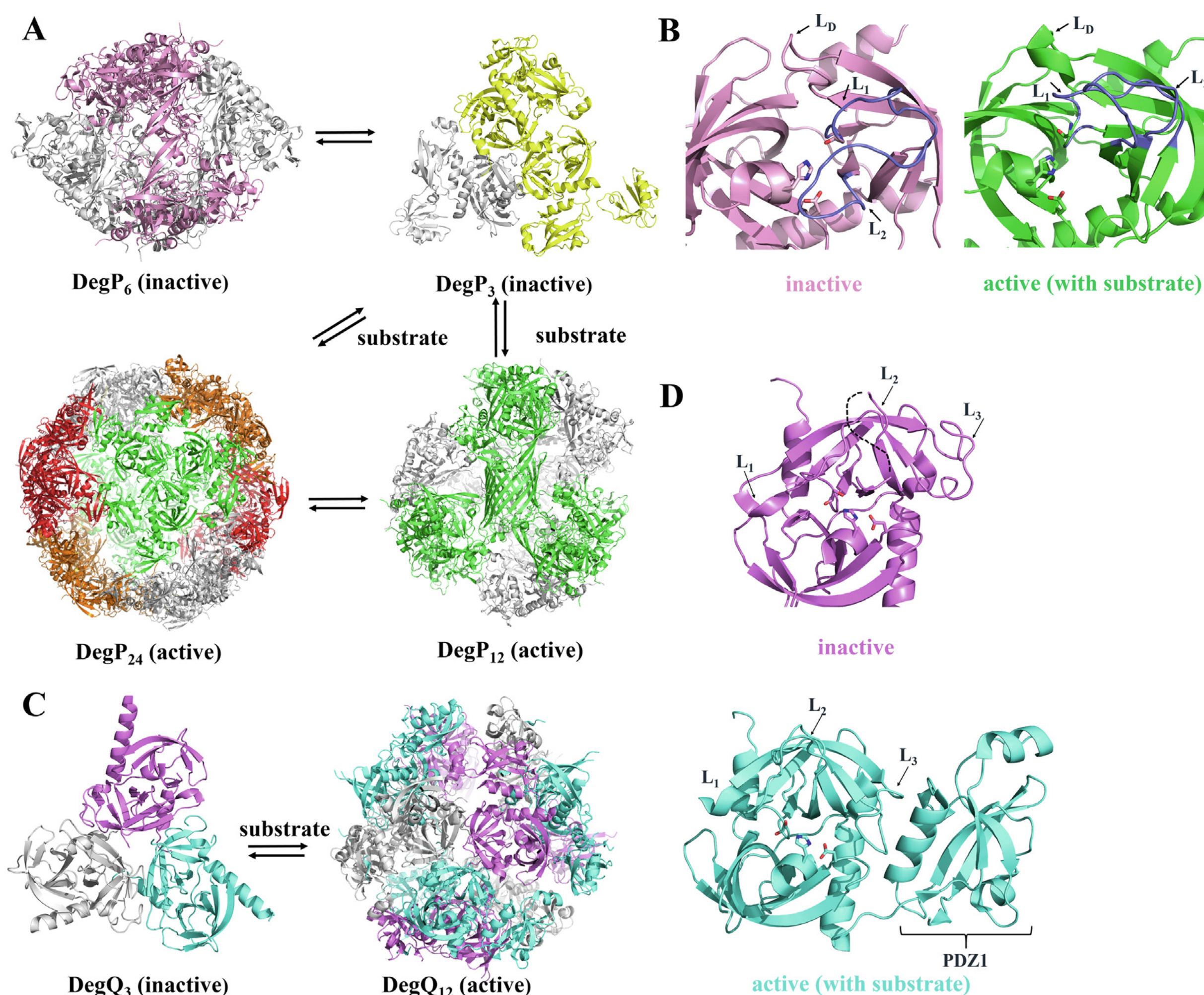

Fig. 5. Oligomerization and activation mechanism of DegP and DegQ. (A) Oligomer and domain structures of $DegP_6$ (1KY9) [3], $DegP_3$ (3OTP) [77], $DegP_{12}$ (2ZLE) [78], and $DegP_{24}$ (3CS0) [78]. On binding substrate, DegP changes from an inactive hexamer to a transient trimer, primed to form 12-mer or 24-mer cages. (B) Subunit interaction in inactive $DegP_6$ (pink) and active $DegP_{12}$ (green). Mechanistically important loops L1, L2, and LD are highlighted, and residues comprising the catalytic triad (Asp 105, His 135, and Ala 210) are represented by sticks. (C) Ribbon diagram of inactive $DegQ_3$ (3STI) and active $DegQ_{12}$ (3STJ) [15]. The trimer is stabilized by packing between protease domains and the four trimers forming a pyramidal 12-mer DegQ. (D) Subunit interactions in active DegQ (cyan) and inactive DegQ (violet). Active site residues His82, Asp112, and Ser187 are represented by sticks.

DegP may also function in a temperature-dependent manner, becoming activated during heat stress [3,31,35]. However, the underlying mechanism of DegP activation by temperature remains unclear. By using a combination of hydrodynamics and NMR spectroscopy methodologies, Harkness *et al.* found that DegP formed oligomeric assemblies of trimer molecules in the absence of substrate. Such trimers were sensitive to temperature and shifted toward larger oligomeric assemblies including 18-mer cage and 24-mer cage as the temperature increased [35].

A similar mechanism is seen in *E. coli* DegQ, although no hexameric resting states has been detected, probably due to its shorter L_A [14]. In the presence of substrate, four DegQ trimers form a 12-mer complex, enclosing an approximately 50 Å internal cavity (Fig. 5C). Electron density corresponding to loops L_2 and L_3 are not well defined in $DegQ_3$, probably due to high loop flexibility. This conformational variability in the active site leads to the catalytic triad being improperly located. In $DegQ_{12}$, substrate-bound PDZ1 domains interact tightly with L_3 to stabilize the active site conformation; L_2 is also well-defined, facilitating formation of a functional catalytic site (Fig. 5D).

Unlike DegP/Q, most DegS-like HtrAs are inner membrane-anchored proteases, which form trimers *in vivo* (Fig. 6A) [5]. Structural analysis indicates that DegS trimerization is mediated solely by the N-terminus and residues β7-β12 of the protease domain. When *E. coli* DegS is in an inactive state, loop L_3 is trapped by the PDZ domain. The hydroxyl of Ser201 interacts with Asn94, which prevents nucleophilic attack of His96, causing inhibitory contacts between PDZ and protease domains (Fig. 6B).

OMP binding by the PDZ domain induces several conformational changes including L_3 release by PDZ, ring flipping of residue His198, and reorientation of Ser201, His96, and Asp126 into an active configuration (Fig. 6B). The PDZ domain is inhibitory in DegS: a mutant protein without PDZ exhibits cleavage activity in the absence of the OMP peptide [7]. However, we recently showed the PDZ domain of *P. aeruginosa* AlgW forms inter-domain contacts to convert and maintain an active catalytic triad [16]. One explanation suggests different inter-domain interaction networks exist in DegS versus AlgW. In AlgW, L_3 maintains interactions with PDZ after activation and L_A exhibits joint angle shifts between active and inactive states (Fig. 6C) [16]. Moreover, *P. aeruginosa* AlgW is the first example of a dual activator-binding DegS-like HtrA where peptide and lipid enhance cleavage activity synergistically [16]. As the synergistic binding of peptide and lipid is a normal feature of many PDZ-containing proteins [70], we propose that such a peptide/lipid-regulatory mechanism may exist in other HtrA proteins.

6. Drug development targeting HtrA proteins

Bacterial proteases are promising targets for the development of new antibiotics. Synthetic inhibitors of ClpXP proteases are potential agents active against *Mycobacterium tuberculosis* and *Staphylococcus aureus* [71,72]. In a recent review, Xue *et al.* briefly summarized the importance of HtrA in bacteria and drug development [73]. We see the HtrA protease as an interesting drug target due to its size, vital biological functions, highly conserved sequence and structure, and the presence within the PDZ domain of a clearly identifiable and highly specific binding site.

A key example is the secreted *Helicobacter pylori* HtrA protein. Perna *et.al.* used fragment-based de-novo design to identify novel lead compounds able to inhibit HtrAs [74]. Further studies identified compounds active *in vivo* able to inhibit proliferation and migration of *H. pylori* (Fig. 7A). Bongard *et al.* reported that modified pyrazolo[1,5-*a*]-1,3,5-triazines were potential *E. coli* DegS inhibitors [19]. They demonstrated these compounds could repress the outer membrane stress response and act synergistically with antibiotics in *E. coli* (Fig. 7A). These inhibitors lost activity in DegSΔPDZ strains, suggesting they primarily target PDZ domains. JO146 [Boc-Val-Pro-Val^P(OPh)$_2$] is a peptide inhibitor of *Chlamydia trachomatis* HtrA. Irreversible analogues of JO146 showed inhibitory activity and are considered potential anti-chlamydial drugs (Fig. 7A) [20,73]. Modifications to the N-cap of these inhibitors only slightly alter their antibacterial activities, suggesting routes to future optimization.

There are two distinct strategies for HtrA inhibitors design: directly targeting the active site or binding the PDZ domain to repress HtrA activation. Most inhibitors contain three components

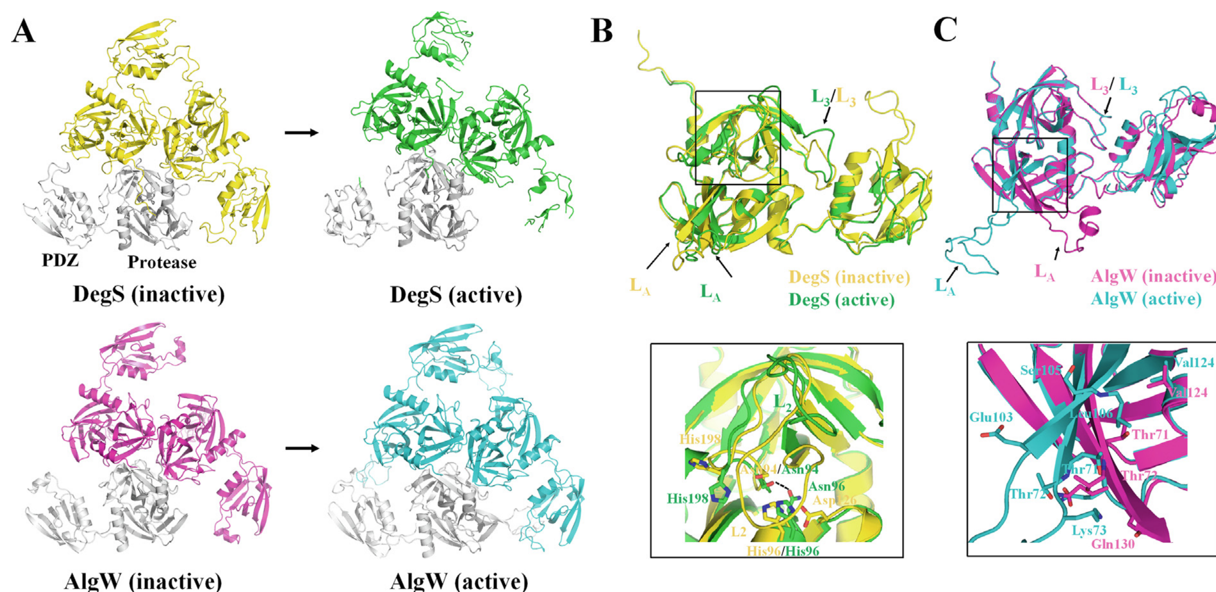

Fig. 6. Activation mechanisms of DegS and AlgW. (A) Ribbon diagram of inactive DegS (PDB: 1TE0) [13], active DegS (PDB: 1SOZ) [5], inactive AlgW (PDB: 7CO7), and active AlgW (PDB: 7CO5) [16]. Both DegS and AlgW are inner membrane proteases and function as a trimer *in vivo*. The trimer has each subunit colored differently. (B) Subunit interaction in active DegS (green) and inactive DegS (yellow). Magnified view of L_2 moving between active and inactivate states. (C) Subunit interaction in active AlgW (blue) and inactive AlgW (pink). Different form of DegS, where subdomain movement is mainly concentrated in L_A (magnified view).

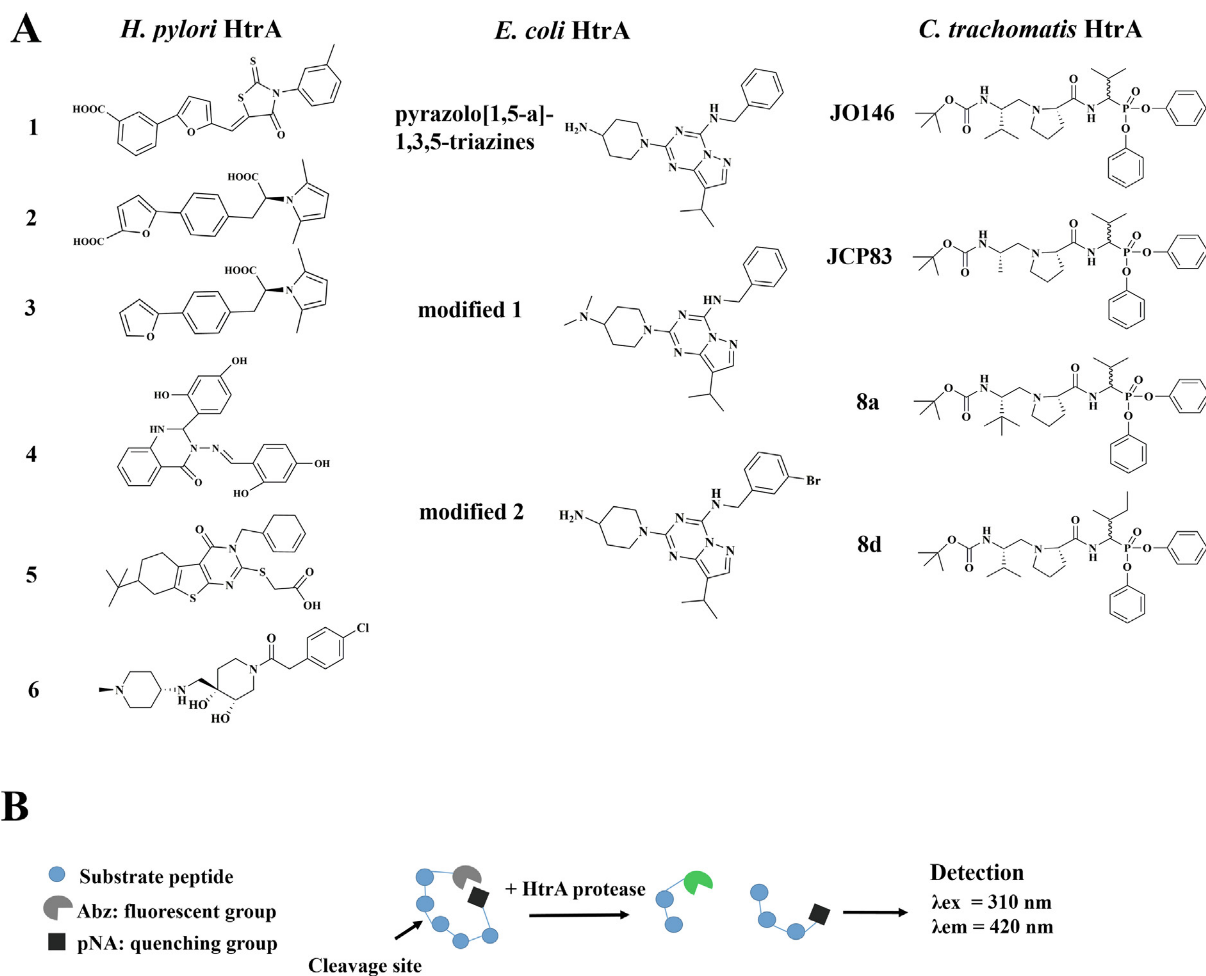

Fig. 7. HtrA Drug development. (A) Structure of representative inhibitors of HtrA from *H. pylori*, *E. coli*, and *Chlamydia trachomatis*. (B) Schematic diagram of the Abz-peptidyl-pNA fluorescence resonance energy transfer (FRET) protocol used for HtrA. Anthraniloyl group fluorescence is quenched by pNA and can be released by HtrA peptide cleavage. Increased fluorescence that can then be monitored using a multiskan spectrum microplate. Enzyme activity curves reflect the effects of small molecule inhibitors on HtrAs.

and are similar in size, for example JO146, which was based on tripeptide VPV. As both protease and PDZ domains can bind short peptides, polypeptide analogues may generate large compound libraries for HtrA screening.

The bottleneck for HtrA inhibitor development is establishing appropriate and efficient high throughput screening (HTS). A sensitive Abz-peptidyl-pNA fluorescence resonance energy transfer (FRET) protocol can quantify the activity of HtrA inhibitors using an *in vitro* assay (Fig. 7B) [16,75,76]. The quenched fluorescent peptide substrate comprises Abz (fluorogenic group), peptides with cleavage sites, and pNA (P-nitroaniline), a known fluorescence quencher. After HtrA cleavage, fluorescence is determined from the absorbance measured at 420 nm (λex = 310 nm, λem = 420 nm), allowing development of a high-volume HTS (Fig. 7B).

7. Challenges and future perspectives

Due to the high mortality arising from both emerging hospital-acquired and community-acquired infections, bacterial diseases have recently gained much attention. Because of their prevalence in prokaryotes and their roles in external stress survival, HtrA proteins are considered interesting targets for the development of antimicrobial agents. However, the screening and development of HtrA inhibitors has just begun and their precise mechanisms of action remain unclear. Many bacterial HtrA proteins are not yet well defined. Their structural and functional diversity needs further study.

HtrA proteins have many roles: stress survival, signal transduction, biofilm formation, and as direct invasion factors. Targeting HtrA in different bacteria could give rise to selective bacteriostatic and inhibition efficiency effects. Avoiding nonspecific binding to human HtrA proteins and other side effects due to off-target activity in cells, tissues, and different host microbiomes is also challenging. This may require studying distinct structure characteristics and regulatory mechanisms within and between prokaryotes and humans. Secreted HtrAs could be used to assay specific immune activity when detecting bacterial infection in clinical trials. By combining HTS with molecular docking, it may be possible to identify new inhibitors of HtrA rapidly and efficiently.

Despite limited success hitherto, HtrA proteins and their inhibitors show remarkable promise for the development of new antimicrobial drugs. While considerable additional research is still required, as outlined here, the future of this discipline promises much of societal value.

CRediT authorship contribution statement

Yingjie Song: Data curation, Formal analysis, Writing-review & editing. **Yitao Ke:** Data curation, Formal analysis, Writing-review & editing. **Mei Kang:** Funding acquisition. The Investigation, Project administration, Software, Supervision, Validation, Visualization,

Writing-original draft. **Rui Bao:** Data curation, Formal analysis, Funding acquisition. Investigation, Project administration, Software, Supervision, Validation, Visualization, Writing-original draft. All authors participated in Writing-review & editing and agreed to the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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