# *In silico* Investigation of Lon Protease as a Promising Therapeutic Target

Authors
**Parisa Asadollahi[1, 2], Iraj Pakzad[1, 2], Sobhan Ghafourian[1, 2], Hossein Kazemian[1, 2], Roohollah Fatahi[3], Nourkhoda Sadeghifard[2], Behrooz Sadeghi Kalani[1, 2]**

Affiliations
1 Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran
2 Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran
3 Faculty of Para Veterinary Medicine, Ilam University,Ilam. Iran

Key words
in silico, Lon protease, *E. coli*, Antitoxin, inhibitory peptides

**received** 25.09.2021
**accepted** 30.11.2021
**published online** 18.01.2022

Bibliography
Drug Res 2022; 72: 180–188
**DOI** 10.1055/a-1713-3137
**ISSN** 2194-9379

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

Correspondence
Nourkhoda Sadeghifard
Professor of Bacteriology
Clinical Microbiology Research Center
Ilam University of Medical Sciences
Pazhuhesh Blvd
Ilam
Iran
Sadeghifard@gmail.com

Behrooz Sadeghi Kalani
Ph.D. of Bacteriology
Department of Medical Microbiology
School of Medicine
Ilam University of Medical Sciences
Pazhuhesh Blvd
Ilam
Iran
Tel.: + 98/918/4656 065
Behroz.sadeghi@gmail.com

**Supplementary Material** is available under https://doi.org/10.1055/a-1713-3137.

**ABSTRACT**

Considering the widespread occurrence of antibiotic resistance, the need for new therapeutic strategies is inevitable. Bacterial proteases are a broad set of enzymes that play a vital role in cell survival, stress response, and pathogenicity. This *in silico* study was aimed to focus on the crucial role of Lon protease in the regulation of toxin-antitoxin systems in *E. coli* and to design inhibitory peptides against the action of this protease. With the help of relevant servers and softwares, the communication network, the evolutionary history, and the interaction of Lon with the corresponding antitoxins were examined, following which the inhibitory peptides were designed against these interactions. The results showed that Lon protease plays a central role in the control of these systems and is a conserved protein, especially among the Enterobacteriaceae family. The docking results of the designed peptides with the Lon protease were significant. This study showed that Lon protease may have the characteristics of a new therapeutic target.

## Introduction

Over the last decade, the issue of antimicrobial resistance has challenged the eradication of bacterial infections [1, 2]. Antibiotic resistance due to mutation or the acquisition of resistance genes may occur regardless of antibiotic exposure. However, the presence of these agents induces a dramatic increase in the incidence of resistant bacteria [3]. This underscores the necessity of designing novel antibacterial agents [4]. Despite many efforts to modify the present antimicrobial drugs, only a few have proven effective against resistant bacteria [4]. One practical approach to overcome this problem is the identification of potential bacterial targets for developing suitable therapeutic agents [4]. Recent studies about the bacterial physiology and behavior have paved the way for the identification of a variety targets, among which Lon protease in *Escherichia coli* strains is an interesting one [5].

Lon protease, the first identified ATP-dependent serine protease with a highly conserved structure, is a homo-oligomer with ATPase and proteolytic domains [6]. Lon has a crucial role in the protein qual-

ity control system, getting involved in the eradication of aberrant and misfolded proteins and the selective degradation of regulatory proteins, including those associated with cell division, capsule synthesis, and SOS response, thereby modulating numerous metabolic and stress response pathways [6, 7]. Lon also contributes to the cleavage of antitoxins in toxin-antitoxin (TA) systems, where the related liberated toxins can inhibit bacterial growth by suppressing translation or replication [7, 8]. Activation of TA systems through antitoxin degradation could result in a variety of phenotypes, but the most frequently observed are those connected with growth inhibition, persistence, programmed cell death, and biofilm formation [7, 9]. The ubiquitousness of these systems among clinical strains and their absence in eukaryotic organisms makes them ideal targets for the development of suitable agents and the subsequent treatment of *E. coli* infections. In general, protein-protein interactions (PPIs) play a vital role in many biological processes and are associated with cancers and infectious diseases, accordingly, targeting PPIs can be a promising therapeutic strategy [10, 11]. Considering the regulatory and central role of Lon protease in the functionality of the TA systems, we aimed to study the bioinformatics of Lon and its interaction with related TA systems, as well as designing interfering peptides for restraining Lon-antitoxin interactions.

# Methods

## Lon and TA system network

In order to predict the interacting protein network associated with Lon and the corresponding TA systems, the STRING's server was used.

## Conserved domains and consensus sequences of the Lon gene

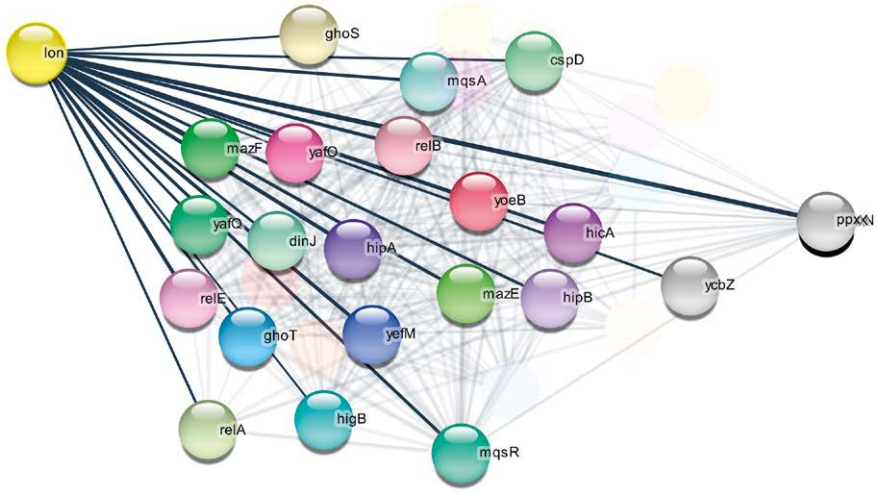
The results of PSI-BLAST were introduced into the Jalview 2.8.1 and the alignment file with the respective gap open cost and gap extension cost of 10.0 and 1.0 was created [12]. Conserved domains were acquired from the Pfam 32.0 database by multiple sequence alignments and hidden Markov models (HMMs) [13].

## Phylogenetic study of the Lon

To study the phylogeny of the Lon protein, the results of the PSI-BLAST with at least 60 % identity were chosen. Following the omission of duplicates and redundant sequences, molecular phylogeny was inspected using Maximum Likelihood method by keeping bootstrap value 200. The evolutionary background was deduced with reference to the JTT matrix-based model. Evolutionary analyses were performed in MEGAX [14].

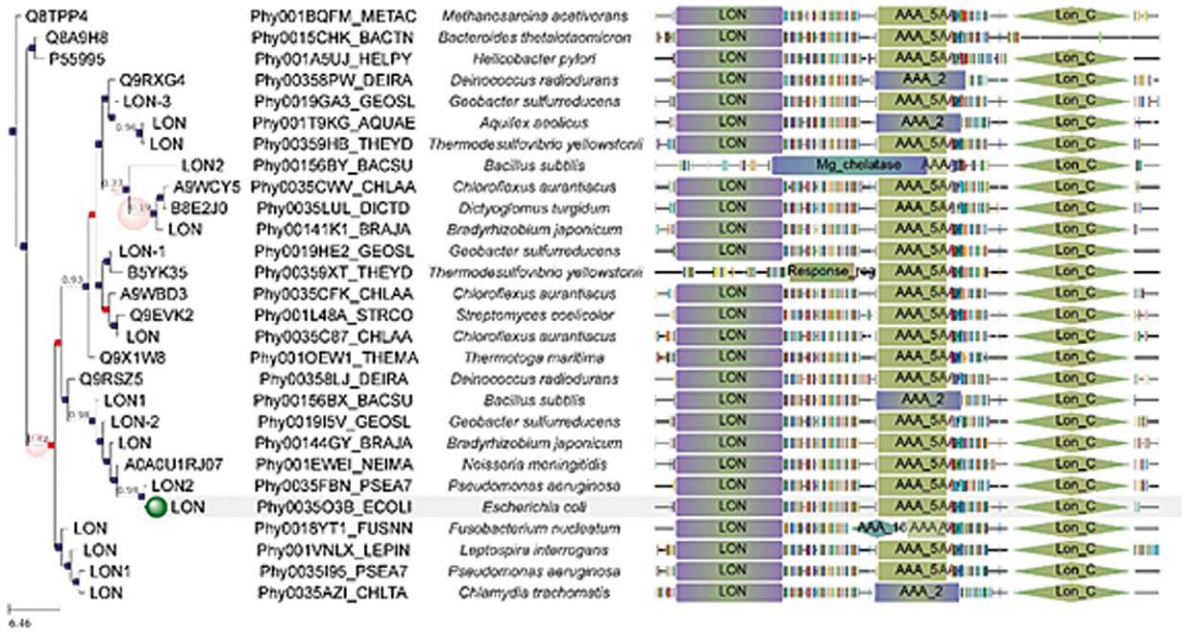
▶ **Table 1** Characteristics of the predicted peptides against Lon/antitoxin interactions.

| TA (PDB code) | Complex inhibited | Peptide sequence | Interface score | Relative interface Score (%) | Binding energy |
|---|---|---|---|---|---|
| 3G7Z | Lon/CcdA | EVARFIEMNGSFADEN | −20.630 | 44.04 | −652.9 |
| 2WIU | Lon/HipB | TLTTFFKILQSLELSMTL | −16.071 | 38.68 | −729.3 |
| 1UB4 | Lon/MazE | DITPENLHENIDWGEP | −29.437 | 49.47 | −704.7 |
| 4FXE | Lon/RelB | PSEALRLMLEYIADNE | −23.234 | 56.72 | −595.3 |
| 3HI2 | Lon/MqsA | VHCEESIMNKEESDAF | −12.090 | 45.89 | −696.1 |
| 2A6Q | Lon/YefM | MSLEEYNSLEETAYLL | −6.267 | 54.42 | −746.6 |




▶ **Fig. 1** Protein-protein interaction network associated with Lon protease and the corresponding ATs using the STRING server. In addition to the antitoxins CcdA, HipB, MazE, RelB, MqsA, and YefM, Lon has controlling roles over several other TA systems in E. coli including HicB and HigA.


| Family | Description | Entry type | Clan | Envelope | | Alignment | | HMM | | HMM length | Bit score | E-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Start | End | Start | End | From | To | | | |
| Lon_C | Lon protease (S16) C-terminal proteolytic domain (shorten) | Domain | CL0329 | 569 | 773 | 570 | 772 | 2 | 204 | 205 | 325.4 | 1.2e-97 |
| LON_substr_bdg | ATP-dependent protease La (LON) substrate-binding domain (shorten) | Domain | CL0178 | 10 | 202 | 10 | 201 | 1 | 207 | 208 | 146.4 | 9.9e-43 |
| AAA | ATPase family associated with various cellular activities (AAA) (shorten) | Domain | CL0023 | 352 | 493 | 352 | 489 | 1 | 128 | 132 | 64.1 | 1.7e-17 |




▶ **Fig. 2** Results of domain and phylum analysis of the Lon protease using PhylomeDB [21]. Lon is conserved among major lineages of the domain bacteria.

## Lon proteases and their interactions with corresponding ATs in E. coli

To date, numerous TA systems have been detected in E. coli however, in this study we concentrated on the dominant TA systems controlled by the Lon protease, the antitoxins component of which include CcdA, HipB, MazE, RelB, MqsA, and YefM [15]. First, the TA and Lon structures were extracted from the protein data bank (PDB) database (▶ **Table 1**). Then, to explicate the interaction between Lon protease and the studied ATs, molecular docking was conducted for the proteolytic domain of Lon and ATs using the ClusPro server [16]. To elucidate the interacting residues, protein complexes with minimum binding energy were selected and envisioned using the LigPlot + software [17].

## Prediction of peptide-mediated interactions

Peptides capable of obstructing Lon/AT interactions in *E. coli* were designed using the Peptiderive server [18]. This server provides linear peptides for a specific protein-protein interaction based on "hot segments", which provides an interface score representative of the binding energy of the protein-peptide complex at that particular position.

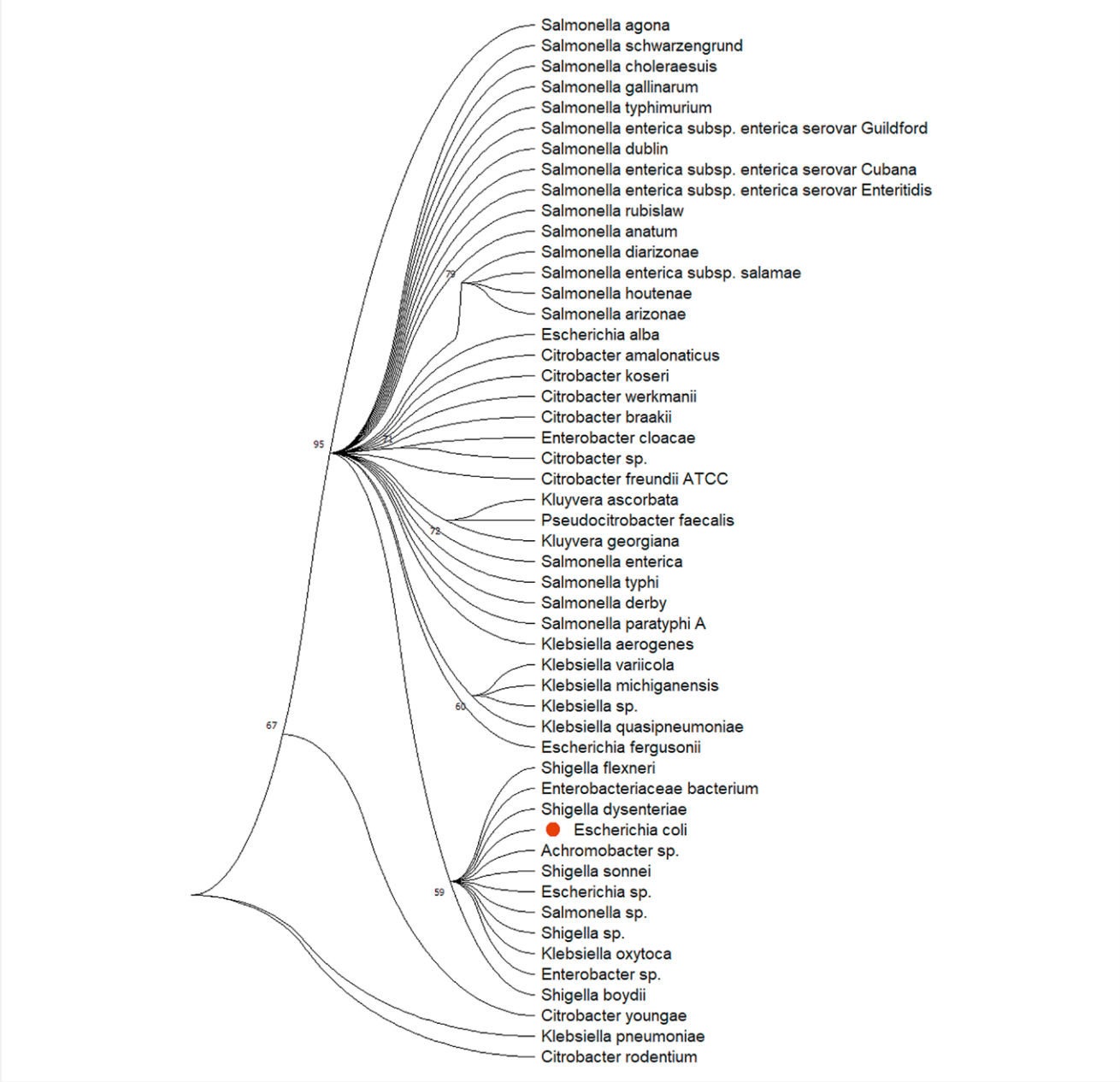
The tertiary structures of the peptides were predicted using the PEP-FOLD server [19], following which the protein-peptide docking was performed using the Cluspro server.

## Visual presentations

Protein-peptide interactions were visualized and recorded using Pymol [20] and LigPlot + software.

## Results and Discussion

Due to the emergence of antibiotic resistance among many bacteria, finding new antimicrobial targets is essential [1]. Bacteria appear to have found effective ways to neutralize antibiotics. Therefore, the study of new targets such as vital enzymes, signaling pathways, efflux pumps, etc. can be an alternative approach [21]. In *E. coli*, as one of the most important pathogenic bacteria, Lon pro-

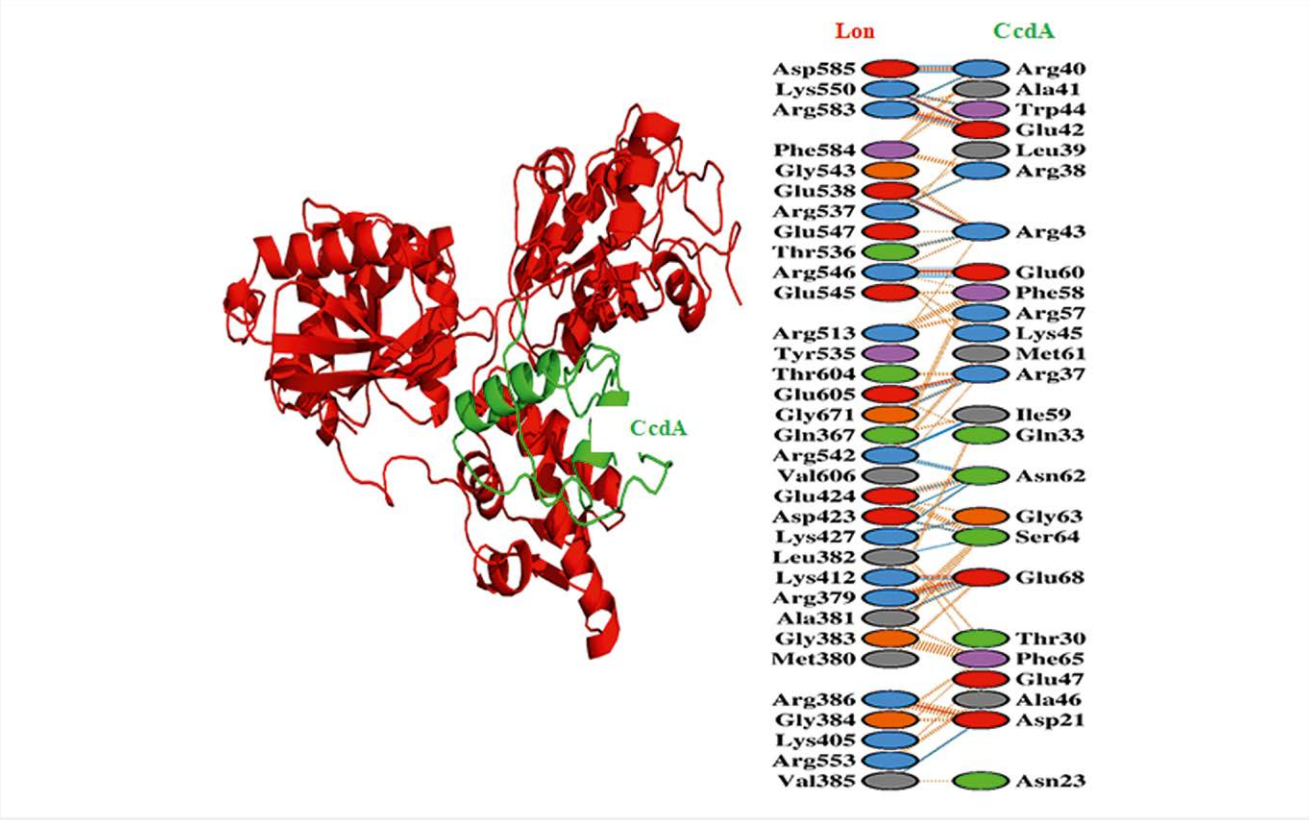



▶ **Fig. 3** Maximum likelihood tree using JTT matrix-based model. Numbers at each node are bootstrap percentages for 200 replicates. Evolutionary analyses were performed in MEGAX.

tease is a vital protein for bacterial growth, metabolism, and survival [6]. One of the interesting mechanisms of this protease is its regulatory role in toxin-antitoxin (TA) systems [8].

TA systems have received much attention in recent years. Various studies have shown that these systems help bacterial survival in stress conditions through different mechanisms including biofilm and persistence development, which lead to chronic and recurrent infections [22]. The activity of these systems is controlled by proteases such as Lon, which break down the antitoxin component in stress conditions to liberate the toxin component. The toxin is thereby released to inhibit bacterial growth via different mechanisms [8].

Today, with the tremendous increase of bio-data in databases and the significant development of bioinformatics and computational tools, it is possible to work more quickly in the in-silico space on the screening, identification, prediction, and design of antimicrobial compounds.




▶ **Fig. 4** Cartoon representation of the 3D structure and interactions of the Lon protease with the CcdA antitoxin. Red color indicates Lon protease and the green color indicates CcdA antitoxin.

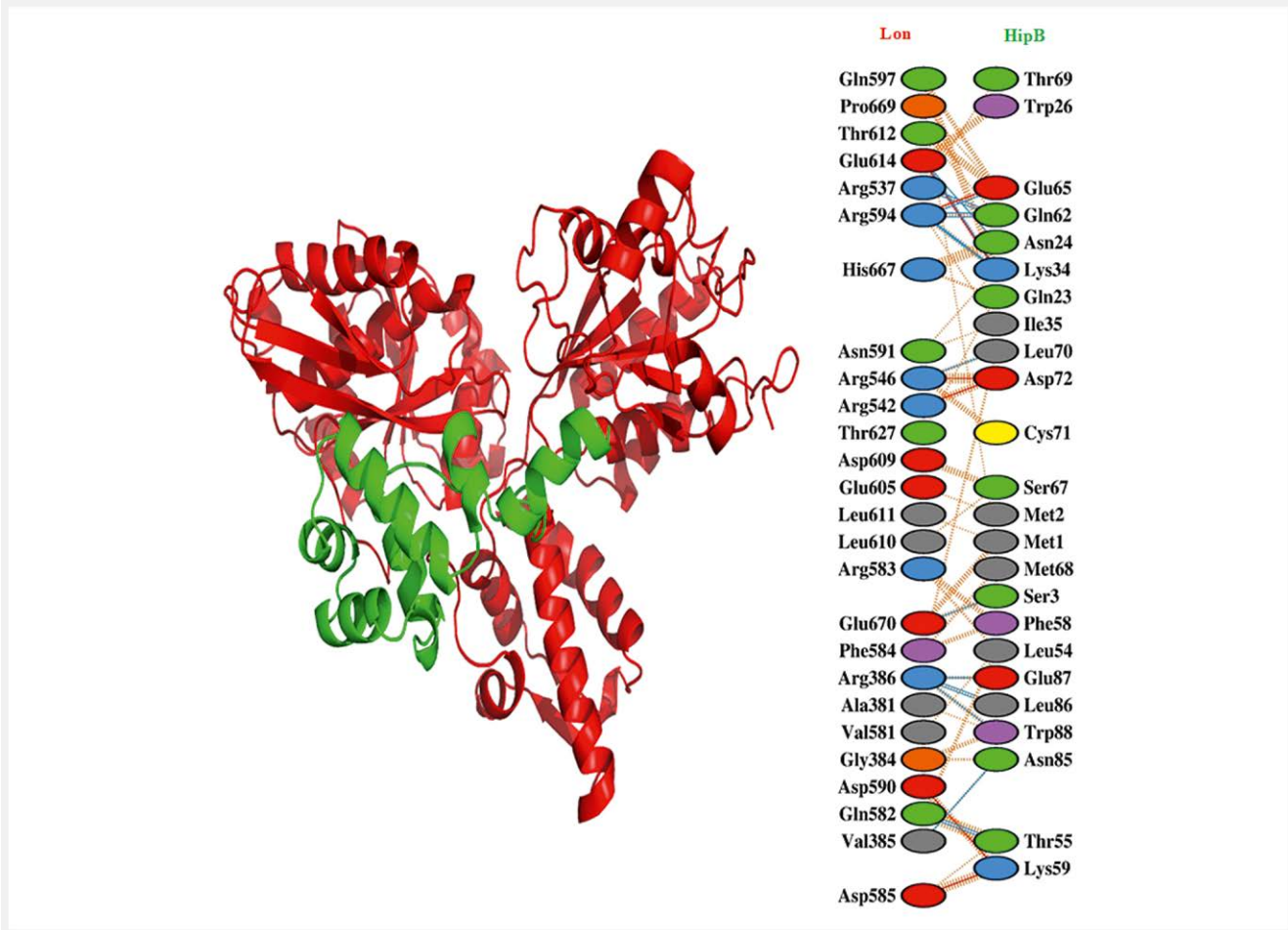
Therefore, this study has focused on the structure, evolution, and regulatory function of the Lon protease of TA systems in *E. coli* as a new antimicrobial target and finally the design of inhibitory peptides to neutralize its regulatory effect. The results of functional connective networks of Lon and the corresponding ATs using the STRING database (▶ **Fig. 1**) showed that in addition to the antitoxins CcdA, HipB, MazE, RelB, MqsA, and YefM, Lon has controlling roles over several other TA systems in E. coli including HicB and HigA, necessitating more studies in this field.

Lon has a major role in controlling the functional network of the systems shown in ▶ **Fig. 1**; along with other proteins that may be involved in this network (▶ **Fig. 1**). Structural analysis of the Lon protease indicated three domains, including the substrate-binding domain, the AAA-rich domain with several cellular activities, and the C-terminal domain with proteolytic activity (▶ **Fig. 2**). Moreover, phylogenetic analysis of Lon indicated its presence in a conserved manner (especially in the C-terminal region) among the Enterobacteriaceae family (▶ **Fig. 3**).

The results of the phylogenetic tree showed that this protease has a common ancestor among the bacteria of this family in terms of evolutionary distance, which has been fully protected over time. Moreover, its homologous can be found in all bacteria known until now (▶ **Fig. 2**) indicating the importance of this protease in bacterial homeostasis; hence being a suitable target for antimicrobial purposes. To investigate how the Lon protease interacts with the studied antitoxins, the docking technique was performed by the ClusPro server and to understand the functionally interacting residues, protein complexes with the lowest binding energy were chosen and visualized using LigPlot + software. The amino acids involved in these interactions are shown in ▶ **Figs. 4–9**.

In recent years, peptide drugs such as natural or synthetic interfering peptides have received much attention due to their physical and chemical properties, and ease of synthesis and handling [11, 23, 24]. Online servers and computational soft wares have been used in this study to design interfering linear peptides (Peptide drive server) to interfere with the Lon/antitoxin interactions. The sequence of these peptides is shown in ▶ **Table 1**.

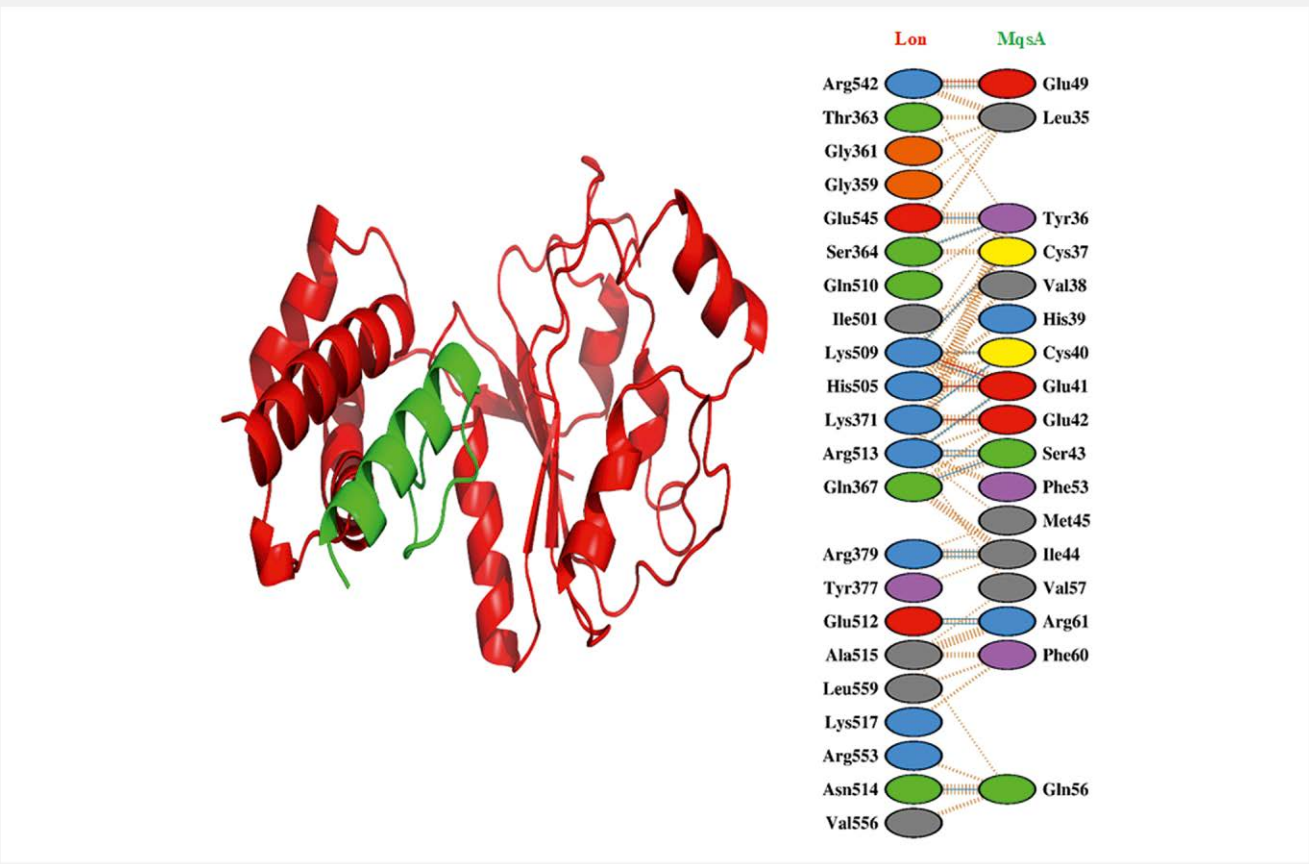
To evaluate the binding energy of these peptides, the 3-D structure of the peptides was first modeled using the PEP-FOLD server (▶ **Fig. 10**) and then docked with the Lon protease using the ClusPro server. The results of this section showed that the linear peptide EVARFIEMNGSFADEN has 16 amino acids in length and can bind to 26 residues of the Lon protease with a binding energy of -652.9, interfering the Lon/CcdA interaction (▶ **Fig. 11a**).




▶ **Fig. 5** Cartoon representation of the 3D structure and interactions of the Lon protease with the HipB antitoxin. Red color indicates Lon protease the green color indicates HipB antitoxin.

CcdA is the antitoxin component of the CcdA/B TA system that is involved in the maintenance of plasmids and the death of plasmid-deficient cells in *E. coli* (Post-segregational killing) [25]. Another peptide designed in this study had the TLTTFFKILQSLELSMTL sequence that could bind to the interface of the Lon/HipB, with a binding energy of -729.3 (▶ **Fig. 11b** ). The *hipB* antitoxin gene is located on the upstream of the hipA/B operon. Studies have shown that this antitoxin plays a role in the formation of *E. coli* biofilm, such that its removal reduces the ability to biofilm formation [26]. MazE/F TA is one of the most well-known and conserved TA systems among bacteria which, in stress conditions, is involved in programmed cell death as well as biofilm formation [27]. To inhibit the regulatory effect of the Lon protease on this system and interfere with the Lon/MazE interaction, the 16-amino acid linear peptide DITPENLHENIDWGEP was predicated by the Peptide Drive server. It should be noted that the linear peptides designed in this study are derived from the amino acid structure of the studied antitoxins, which mimic the behavior of antitoxins in binding to the Lon protease. Information on other peptides designed to interfere with the interaction of the Lon protease with RelB, MqsA, and YefM antitoxins are shown in ▶ **Table 1** and ▶ **Figs. 11a–f**. In general, the docking results of the designed peptides to the Lon protease and their binding energy have proven encouraging as means of interfering with these TA systems.

The vital roles of Lon and its homologues among bacteria have made this protease an attractive antimicrobial target for researchers. In a study in 2019, M. Babin et al. examined the effects of different hybrid peptides on the Lon protease inhibition in *E. coli*. They screened various peptide compounds and showed that boronic acid has efficient Lon-binding and -inhibitory capacity. Their results showed that interfering with this protease accelerates the UV induction of bacterial filamentous structure and also reduces bacterial tolerance to the antibiotic ciprofloxacin [28].




▶ **Fig. 8** Cartoon representation of the 3D structure and interactions of the Lon protease with the MqsA antitoxin. Red color indicates Lon protease and the green color indicates MqsA antitoxin.

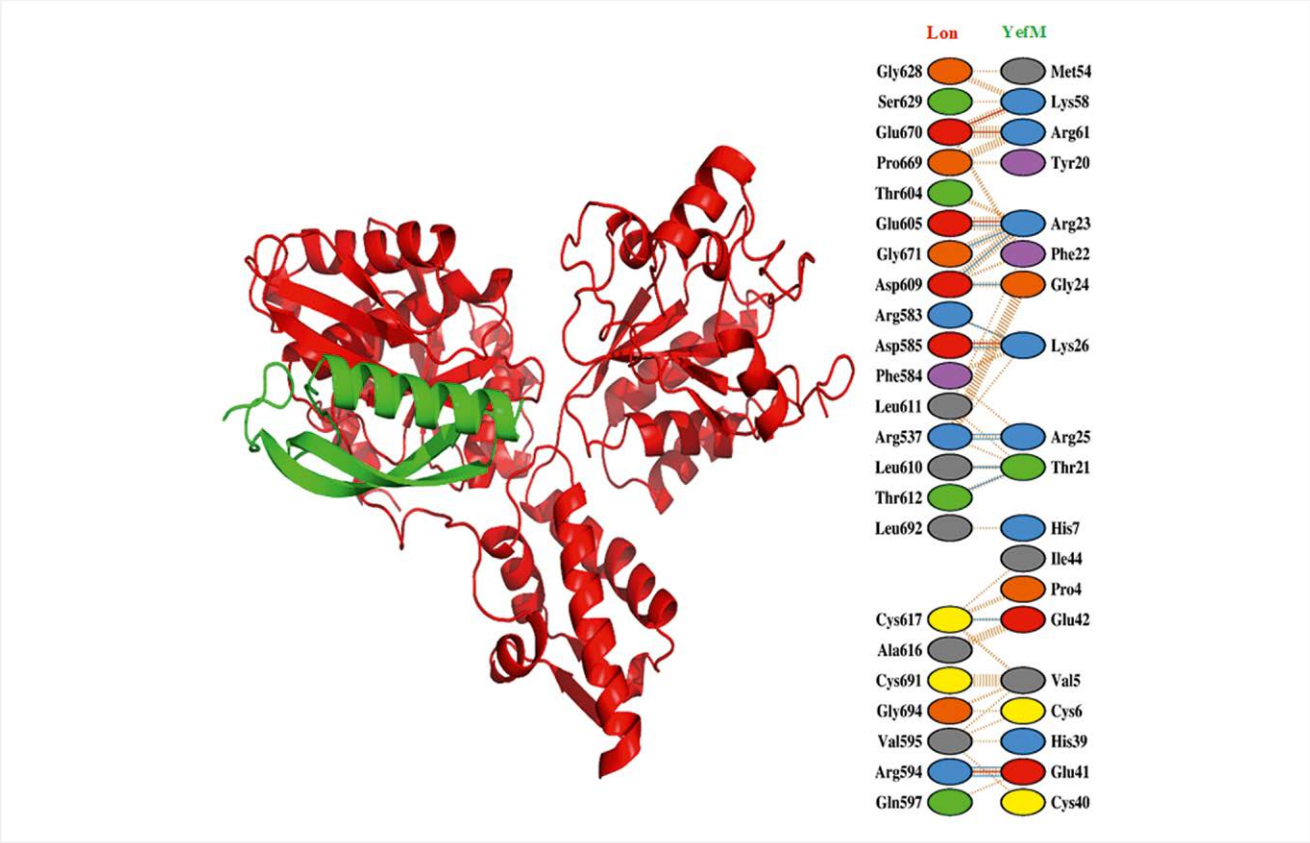
In 2020, in an *in silico* study on TA systems and ClpP regulatory protease in *Listeria monocytogenes*, Mohammadzadeh et al. showed that the interaction between the studies TA systems and the ClpP regulatory protease could be a new target for antimicrobial peptides. In that study, they predicted linear peptides of 10 to 16 amino acids with ClpP-binding energies of −455 to −907 and stated that these peptides could eventually inhibit or reduce the formation of persister cells in *L. monogytogenes* [29].

In another study, Suredr et al. designed the linear peptides ELAAIRHRCA and AYPYESEAER to inhibit the TA systems VapB/C and MazE/F in *Mycobacterium tuberculosis*, respectively. They declared that these peptides could be new therapeutic compounds against this bacterium given that TA systems are not present among Eukaryotic cells [30].

Peptide-based therapies are being developed because of their ease of design and production, and their encouraging properties such as being highly efficient, selective and well-tolerated by the host [11]. In general, the results of this study showed useful information about the structure and binding properties of the Lon protease and its corresponding antitoxins. Lon, as a central regulatory protease, plays crucial roles in bacterial survival and has characteristics that make it a suitable therapeutic target against antibiotic-resistant bacteria including *E. coli*. The design and use of peptides to interfere and inhibit PPIs in bacteria can be an interesting platform for investigating and outlining new antimicrobial approaches.

## Conflict of interest

The authors declare that they have no conflict of interest.




▶ **Fig. 9** Cartoon representation of the 3D structure and interactions of the Lon protease with the YefM antitoxin. Red color indicates Lon protease and the green color indicates MqsA antitoxin.

## References


[1] Reverter M et al. Aquaculture at the crossroads of global warming and antimicrobial resistance 2020; 11: 1–8

[2] Hendriksen RS et al. Global monitoring of antimicrobial resistance based on metagenomics analyses of urban sewage 2019; 10: p 1–12

[3] Boolchandani M, D'Souza AW, Dantas GJNRG. Sequencing-based methods and resources to study antimicrobial resistance 2019; 20: 356–370

[4] Spaulding CN et al. Precision antimicrobial therapeutics: the path of least resistance? 2018; 4: 1–7

[5] Arends J et al. An integrated proteomic approach uncovers novel substrates and functions of the Lon protease in Escherichia coli 2018; 18: 1800080

[6] Tsilibaris V, Maenhaut-Michel G, Van Melderen L.J.R.i.m. Biological roles of the Lon ATP-dependent protease 2006; 157: 701–713

[7] Page R, Peti W.J.N.c.b. Toxin-antitoxin systems in bacterial growth arrest and persistence 2016; 12: 208–214

[8] Song S, Wood T.K.J.A.b. Toxin/antitoxin system paradigms: toxins bound to antitoxins are not likely activated by preferential antitoxin degradation 2020; 4: 1900290

[9] Ghafourian S et al. Toxin-antitoxin systems: classification, biological function and application in biotechnology 2014; 16: 9–14

[10] Ezraty B et al. Oxidative stress, protein damage and repair in bacteria 2017; 15: 385

[11] Cunningham AD, Qvit N, Mochly-Rosen D.J.C.o.i.s.b. Peptides and peptidomimetics as regulators of protein–protein interactions 2017; 44: 59–66

[12] Waterhouse AM et al. Jalview Version 2—a multiple sequence alignment editor and analysis workbench 2009; 25: 1189–1191

[13] Finn RD et al. The Pfam protein families database: towards a more sustainable future 2016; 44: D279–D285

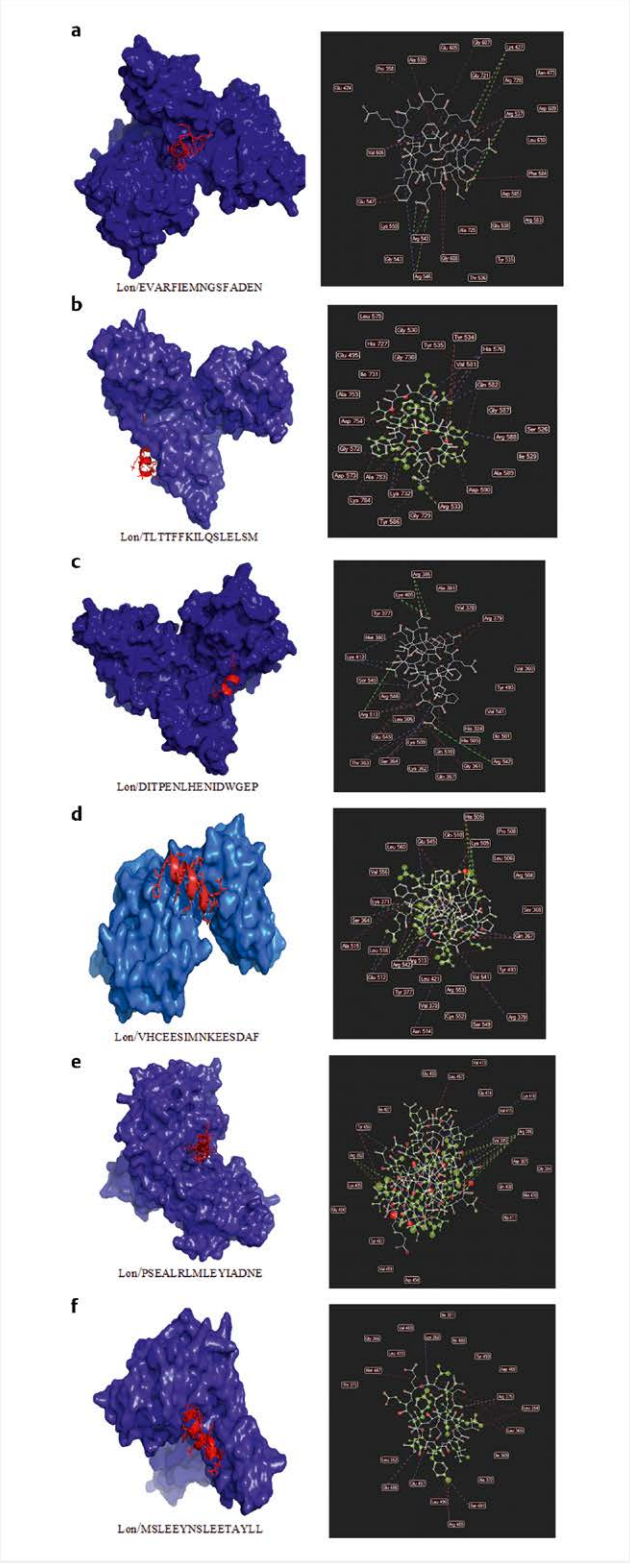
[14] Kumar S et al. MEGA X: molecular evolutionary genetics analysis across computing platforms 2018; 35: 1547–1549

[15] Yamaguchi Y, Inouye MJNRM. Regulation of growth and death in Escherichia coli by toxin–antitoxin systems 2011; 9: 779–790

[16] Kozakov D et al. The ClusPro web server for protein–protein docking 2017; 12: 255

[17] Laskowski RA, Swindells MB. LigPlot + : multiple ligand–protein interaction diagrams for drug discovery. 2011 ACS Publications;

[18] Sedan Y et al. Peptiderive server: derive peptide inhibitors from protein–protein interactions 2016; 44: W536–W541




▶ **Fig. 11** Molecular docking (left panels) and interacting residues (right panels) between the derived peptides and the Lon protease residues.

[19] Lamiable A et al. PEP-FOLD3: faster de novo structure prediction for linear peptides in solution and in complex 2016; 44: W449–W454

[20] DeLano W.L.J.C.N.o.p.c. Pymol: An open-source molecular graphics tool. 2002; 40: 82–92

[21] Culp E, Wright G.D.J.T.J.o.a. Bacterial proteases, untapped antimicrobial drug targets 2017; 70: 366–377

[22] Narimisa N et al. Effects of sub-inhibitory concentrations of antibiotics and oxidative stress on the expression of type II toxin-antitoxin system genes in Klebsiella pneumoniae 2020; 21: 51–56

[23] Magana M et al. The value of antimicrobial peptides in the age of resistance 2020

[24] Kazemzadeh-Narbat M et al. Strategies for antimicrobial peptide coatings on medical devices: a review and regulatory science perspective 2021; 41: 94–120

[25] Wu AY, Kamruzzaman M, Iredell J.R.J.P.o. Specialised functions of two common plasmid mediated toxin-antitoxin systems, ccdAB and pemIK, in Enterobacteriaceae 2020; 15: e0230652

[26] Xu J et al. Functional investigation of the chromosomal ccdAB and hipAB operon in Escherichia coli Nissle 1917 2020; 104: 6731–6747

[27] Wen Y et al. Toxin–Antitoxin systems: their role in persistence, biofilm formation, and pathogenicity 2014; 70: 240–249

[28] Babin BM et al. Leveraging peptide substrate libraries to design inhibitors of bacterial Lon protease 2019; 14: 2453–2462

[29] Mohammadzadeh R et al. In silico insight into the dominant type II toxin–antitoxin systems and Clp proteases in Listeria monocytogenes and designation of derived peptides as a novel approach to interfere with this system 2020; 26: 613–623

[30] Sundar S et al. In Silico Derived Peptides for Inhibiting the Toxin–Antitoxin Systems of Mycobacterium tuberculosis: Basis for Developing Peptide-Based Therapeutics 2019; 25: 1467–1475