Study on Caseinolytic Proteases (Clp) of Pathogenic *Leptospira interrogans*

Thesis Submitted
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

by

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Under the Supervision of Dr. Manish Kumar

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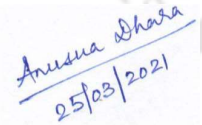
Date

25 March 2021

Declaration

This is to certify that the thesis entitled "Study on Caseinolytic Proteases (Clp) of Pathogenic *Leptospira interrogans*" submitted by me to the *Indian Institute of Technology Guwahati* for the award of the degree of Doctor of Philosophy (Ph.D.), is a bonafide work carried out by me under the supervision of Dr. Manish Kumar. The content of this thesis, in full or in parts, has not been submitted to any other University or Institute for the award of any degree or diploma. I also wish to state that to the best of my knowledge and understanding nothing in this report amounts to plagiarism.

Signed: 

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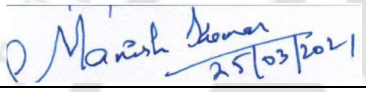
Certificate

This is to certify that the thesis entitled "Study on Caseinolytic Proteases (Clp) of Pathogenic *Leptospira interrogans*", submitted by Anusua Dhara (156106044), a doctorate student in the *Department of Biosciences and Bioengineering*, *Indian Institute of Technology Guwahati*, for the award of the degree of Doctor of Philosophy, is a record of an original research work carried out by her under my supervision and guidance. The thesis has fulfilled all requirements as per the regulations of the institute and in my opinion has reached the standard needed for submission. The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree or diploma.

Signed: 

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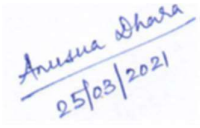
Sincerely

Anusua Dhara



Abstract

The caseinolytic proteases (Clps) highly conserved large oligomeric protein complexes found in prokaryotes and eukaryotes. These complexes are generally involved in maintaining cellular homeostasis as well as virulence regulation. In this study, we aimed at characterizing the Clps of pathogenic *Leptospira in vitro* that might play an essential role in bacterial survival and virulence. Bioinformatic analysis of the genome of the sequenced strains of *Leptospira interrogans* shows the presence of the *clp* system that includes *clpA, clpB, clpC, clpX,* two isoforms of *clpP- clpP1* and *clpP2, clpS, clpY,* and *clpQ*. Based on the *in silico* information, we selected the highly conserved caseinolytic protease P (ClpPs)- ClpP1 and ClpP2 and the cognate ATPase chaperone ClpX for further studies. We have initially characterized the leptopiral ClpP isoforms along with ClpX by various biochemical and biophysical studies. The ClpP isoforms- LepClpP1 and LepClpP2 were in themselves inactive against small peptide substrates but were rendered functionally active when they were mixed. The LepClpP isoforms mixture showed optimum activity at a stoichiometric ratio of 1:1, suggesting a heterocomplex formation. We show that this heterocomplex is a tetradecameric structure and is hypothesized to form two stacked heptamer rings. We have also characterized the ATPase chaperone LepClpX and found that the LepClpP heterocomplex can only degrade larger protein substrates exclusively in the presence of LepClpX in an energy-dependent manner.

Nevertheless, the pure LepClpP1 and LepClpP2 could not be stimulated by LepClpX and ATP to degrade the large protein substrates. Notably, on mutating the serine residue of the catalytic triad of either LepClpP1 or LepClpP2, the heterocomplex became inactive against peptides or protein substrates. Further investigation on the acyldepsipeptide antibiotic (ADEP) mediated activation of LepClpP1P2 heterocomplex revealed that the chemoactivation of ClpP is conditional on the duration of the self-compartmentalization of each of the LepClpP isoforms. We propose a second interaction site of ADEP in the LepClpP heterocomplex, hinted at by the allosteric activation of the LepClpP1P2^{S97A}, an otherwise inactive complex. Antibiotic ADEP also hampered the growth of *L. interrogans in vitro* and elongated the morphology of the spirochete. ClpPs, being conserved in most prokaryotes, including pathogen *L. interrogans*, has shown to be a promising drug target.

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Chapter 1

1.1 LEPTOSPIRA AND LEPTOSPIROSIS

Leptospirosis is an emerging public health problem, particularly in large urban centers of developing countries, and has become the most widespread zoonosis globally. It is caused by spirochetes belonging to the genus *Leptospira*, comprising both saprophytic and pathogenic species. Leptospirosis has a broad geographical distribution, affecting rural and urban areas of tropical, subtropical, and temperate regions. The disease's clinical manifestations can range from mild flu-like illness to a severe disease form known as Weil's syndrome. Acute leptospirosis is characterized by jaundice, renal and hepatic failure, pulmonary distress, and hemorrhage leading to death. The disease outbreaks in developed countries are usually associated with occupational exposure, tourism, or sporting events. However, the disease's significant burden is carried by developing countries with half a million cases reported yearly and a mortality rate ranging from 5 to 10% (Victoriano et al. 2009, Torgerson et al. 2015, McBride et al. 2005, Evangelista and Coburn 2010). Each year, there are significant economic losses due to reproductive disorders in cattle, sheep, pigs, and horses suffering from leptospirosis (Ellis 2015). Despite the severity of leptospirosis and its global importance, the *Leptospira* pathogenesis's molecular mechanisms are not well understood, possibly due to difficulty studying the causal agent by reverse genetics approach (Picardeau 2015). Identifying the *Leptospira* virulence factors and characterization of their activity is particularly important for understanding the disease's mechanisms.

1.2 BIOLOGY OF LEPTOSPIRA

1.2.1 Classification of *Leptospira*

Leptospires are spirochetes that include both saprophytic and pathogenic species comprising the genus *Leptospira*, which belongs to the family *Leptospiraceae*, order Spirochaetales (Faine 1994). Traditional classification specified that all saprophytic species belonged to *Leptospira biflexa*, while *L. interrogans* were included in the pathogenic species (Kmety and Dikken 1993, Plank and Dean 2000, Bharti et al. 2003). Based on DNA hybridization analysis, the currently used classification indicates at least 19 species (13 pathogenic and six saprophytic) (Adler and de la Pena Moctezuma 2010, Levett 2001, Brenner et al. 1999). Seven of these species: *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilli, L. kirschneri,* and *L. alexanderi,* are identified as the primary agents of leptospirosis (Ahmed et al. 2006).

All the recognized *Leptospira* species are divided into 24 serogroups and 250 serovars (Palaniappan et al. 2007) based on the expression of surface-exposed lipopolysaccharide (LPS) (Adler and de la Pena Moctezuma 2010). The structural differences in the carbohydrate moiety of the LPS determine the antigenic diversity of the serovar groups, and serovars containing overlapping antigenic determinants are classified into a larger serogroup. Phylogenetic analyses of 16S rRNA genes suggest that *Leptospira* species are clustered into three groups: pathogenic, saprophytic, and intermediate (Perolat et al. 1998, Levett et al. 2006) (**Figure 1.1**).

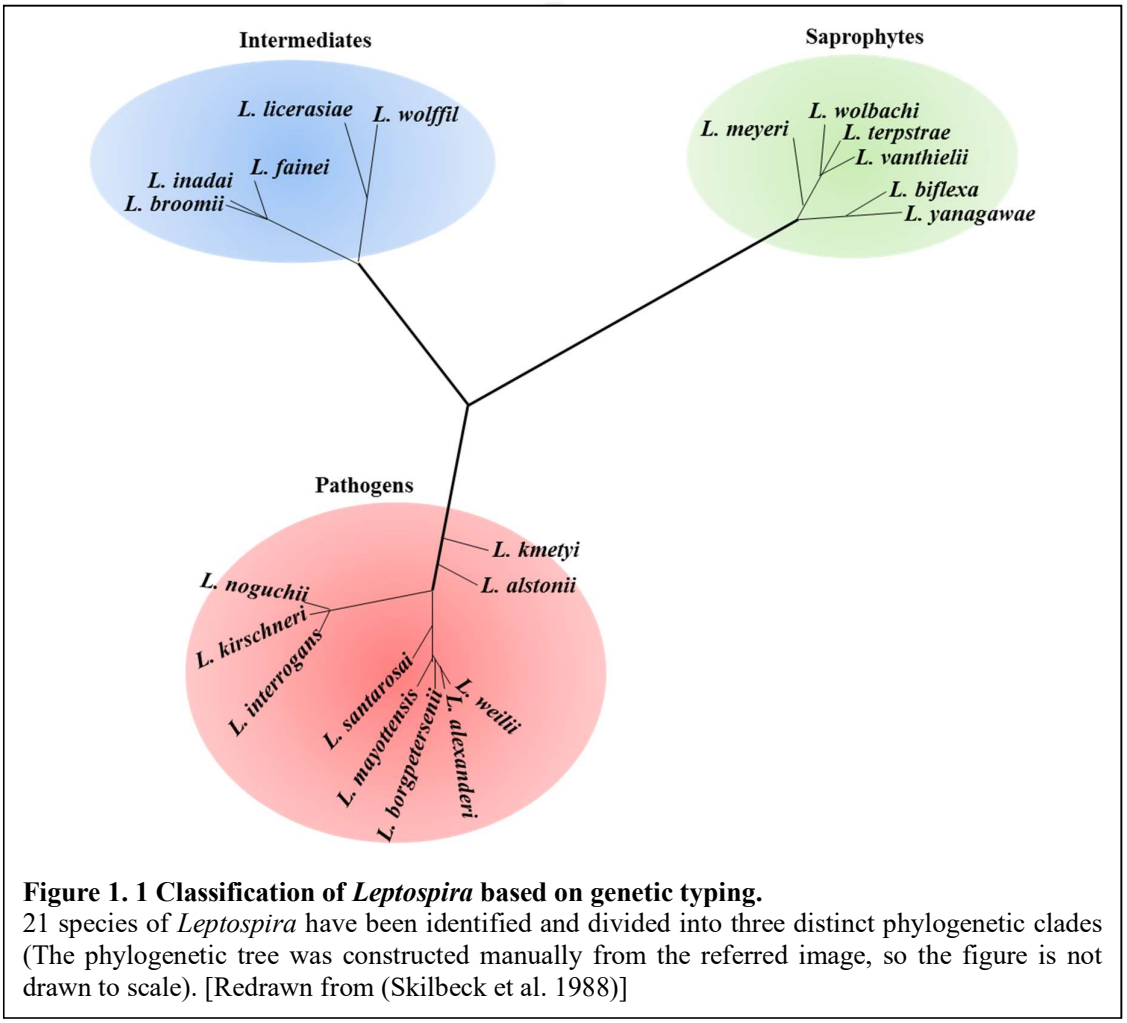

Figure 1. 1 Classification of *Leptospira* based on genetic typing.
21 species of *Leptospira* have been identified and divided into three distinct phylogenetic clades (The phylogenetic tree was constructed manually from the referred image, so the figure is not drawn to scale). [Redrawn from (Skilbeck et al. 1988)]

Recently, the *Leptospira* genus has been re-classified into two major clades or groups and four subclades based on comparative genetics (Vincent et al. 2019). The study has now identified a total of 64 species in the *Leptopira* genus. The major clade "Saprophytes" (clade S) group the species isolated in the natural environment and not responsible for infections. The other clade "Pathogens" (clade P) group all the species responsible for infections in humans and/or animals, and environmental species for which the virulence status has not been proven. The two clades are further subdivided in two subclades each -the subclade P1 (formerly described

as the pathogen group), P2 (formerly described as the intermediate group), S1 (formerly described as the saprophyte group), and S2 (the new subclade).

1.2.2 Cell Biology

Leptospires are thin, helically-coiled, highly motile spirochetes of 6-20 µm in length and can be distinguished morphologically from other spirochetes owing to their unique hook or question-mark shaped ends **(Figure 1.2)**. The leptospires have surface characteristics that share features of both Gram-positive and -negative bacteria. The double-membrane and the presence of LPS are reminiscent of Gram-negative bacteria, while the close association of the cytoplasmic membrane with the murein cell wall is a feature of Gram-positive envelope structure (Ko et al. 2009, Levett 2001, Haake 2000, Vijayachari et al. 2008).

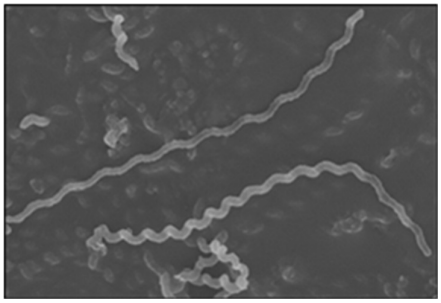

Figure 1. 2 Scanning electron micrograph of *Leptospira interrogans* (20,000× magnification). The electron micrograph shows the elongated structure and helically coiled shape of the bacteria. The characteristic hooked ends can also be seen in the micrograph. (Image courtesy: Md. Saddam Hussain, Ph.D Research scholar of our lab)

1.2.3 *In vitro* cultivation

Leptospires are relatively slow-growing obligate aerobes in both liquid and solid media with an optimal growth temperature of 28-30˚C. They grow in simple media enriched with vitamins B1 and B12, long-chain fatty acids, and ammonium salts (Faine 1999, Adler and de la Pena Moctezuma 2010, Murray et al. 2015, Levett 2001). Long-chain fatty acids are utilized as the sole carbon source and are metabolized by the β-oxidation pathway. The growth of leptospires in media containing serum or albumin and in protein-free synthetic media has also been described. The most widely used medium is based on oleic acid, 1% bovine serum albumin, and polysorbate (Tween 80- a source of long-chain fatty acids) medium EMJH (Ellinghausen-McCullough-Johnson-Harris). Contamination of the medium is prevented by autoclaving both the water and base medium used for preparation, the addition of 5-fluorouracil, and antibiotics such as nalidixic acid or rifampicin (Levett 2001, Faine 1999, Adler and de la Pena Moctezuma 2010) and filter sterilization. Liquid cultures are checked for the growth of the bacteria 3-4 days after inoculation and need to be subcultured after 7-21 days (Bharti et al. 2003).

Pathogenic strain *L. interrogans* can also survive in low-nutrient conditions in the environment, such as moist soil and freshwater for long periods, with salt concentration, pH, and viscosity as critical factors (Trueba et al. 2004). However, *L. borgpetersenii* does not survive outside the host because the strain undergoes loss of critical genes necessary for survival outside the host, thus limiting its transmission through direct host-to-host contact (Bulach et al. 2006). Leptospires can aggregate to form a biofilm, and biofilm formation is proposed to be one of the survival mechanisms employed by the spirochetes to survive in environmental niches (Ristow et al. 2008).

1.3 TRANSMISSION AND MAINTENANCE OF LEPTOSPIROSIS

Leptospirosis is presumed to be one of the most widespread zoonoses in the world. The leptospiral infection source in humans can be either through direct or indirect contact with infected animals' urine. The prevalence of infection is much higher in warm-climate countries than in temperate regions. This is expected due to the prolonged survival of leptospires in nature in warm and damp conditions open doors for human exposure. The occurrence of leptospirosis is seasonal peaking in summer or fall in temperate areas, where the temperature is the restricting variable in the survival of leptospires, and rainy seasons in tropical areas, where quick desiccation would somehow or another avoid survival (Levett 2004).

Leptospires penetrate the body through abrasion or cuts in the skin or the conjunctiva. The waterborne transmission has been archived; point-pollution of water supplies has brought about a few leptospirosis cases (Levett 2001). Inhalation of water or aerosols additionally may bring about contamination through the mucous membranes of the respiratory tract. Seldom, the disease may occur after animal bites. Animals, including man, are the maintenance hosts or incidental (accidental) hosts of leptospirosis. A maintenance host is defined as a species in which the infection is endemic and naturally exchanged from animal to animal via direct contact (Ko et al. 2009). The disease is maintained in the environment by chronic infection of renal tubules of the maintenance hosts. Leptospires colonize the renal tubules of maintenance host species such as dogs, rats, and cattle. They are excreted via urine into the environment and contaminate soil, surface water, streams and rivers. Maintenance hosts are typically asymptomatic while accidental hosts can suffer a wide range of clinical manifestations (Rojas et al. 2010). The most essential maintenance hosts are small mammals, which may transmit contamination to domestic farm animals- cattle, and pigs, dogs and humans (**Figure 1.3**).

The degree to which contamination is transmitted relies on many factors, including climate, population density, and the extent of contact amongst maintenance and accidental hosts. There

is some degree of host-adaptation. Different rodent species may be reservoirs of distinct serovars, but rats are generally maintenance hosts of the related serovars Icterohaemorrhagiae or Copenhageni, and mice of serovar Ballum. Domestic animals are also maintenance hosts; dairy cattle may harbor serovars Hardjo and Pomona, pigs Pomona, Tarassovi or Bratislava, and dogs serovar Canicola (Levett 2001). The maintenance hosts and the serovars they carry can be distinctly variant throughout the world. Knowledge of the prevalent serovars and their maintenance hosts is important in understanding the epidemiology and implementing control of the disease in any region (Levett, 2004).

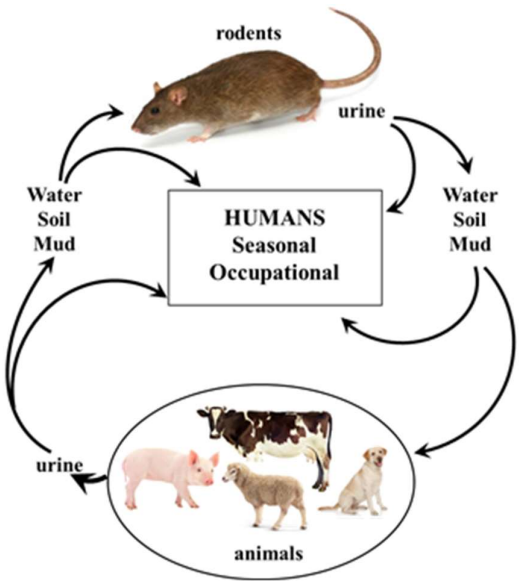

Figure 1. 3 Cycle of leptospirosis
Leptospirosis infection is spread by mammalian species that excrete *Leptospiral* pathogens in their urine and serve as reservoirs for their transmission. Humans are affected either by direct contact with the reservoir animals or by accidental exposure to environmental surface contaminated with their urine. [Soure of the image: (Faine 1999)]

Human infections are acquired through occupational or recreational exposures. Occupation is a major risk factor for humans. Direct contact with infected animals is accounted for most infections in farmers, veterinarians, abattoir workers, meat inspectors, rodent control workers, and other occupations requiring handling of such animals. Indirect contact can concern sewer workers, miners, soldiers, septic tank cleaners, fish farmers, canal workers, sugarcane cutters, rice field workers, etc. There are also significant risks associated with water sports' recreational exposures, as leptospirosis is mostly waterborne disease, including swimming, canoeing, freshwater fishing, rafting (Levett 2001, Bharti et al. 2003).

1.4 CLINICAL MANIFESTATIONS OF LEPTOSPIROSIS

Leptospirosis is a systemic disease of humans and domestic animals, mainly dogs, cattle, and swine, characterized by fever, renal and hepatic insufficiency, pulmonary manifestations, and reproductive failure. Clinical signs are quite variable; most cases are probably apparent and associated with host-adapted serovars such as Canicola in dogs, Bratislava in horses and pigs, Hardjo in cattle and Australis, and Pomona in pigs (Ellis et al. 1986, Andre-Fontaine 2006, Bernard 1993, Grooms 2006). However, other serovars can be involved in more severe disease. Four syndromes have been identified in dogs: icteric, hemorrhagic, uremic (Stuttgart disease), and reproductive (abortion and premature or weak pups). Typical leptospirosis in dogs may present with fever, jaundice, vomiting, diarrhea, intravascular disseminated coagulation, uremia caused by renal failure, hemorrhages, and death (Bolin 1996). In cattle and pigs, leptospirosis signs include reproductive failure, abortion, stillbirths, fetal mummification, weak piglets or calves, and agalactia. A chronic manifestation of leptospirosis is commonly seen in horses as recurrent uveitis (Rohrbach et al. 2005) but is not unique to this species and may also be occasionally diagnosed in humans.

Leptospirosis may present a wide range of clinical manifestations in humans; severity depends on the *Leptospira* strain or serovar involved, inoculum size for at least some strains, and the age, health, and immunological competence of the patient. Many of the documented cases are mild and self-limited and may be hard to distinguish from other infectious diseases until an accurate diagnosis of leptospirosis is made. The disease manifestation in humans is variable, ranging from 1 day to 4 weeks after exposure, and in survivors, the infection can last for months (Plank and Dean 2000). Infection ranges from a mild, influenza-like illness to a severe infection with renal and hepatic failure, pulmonary distress, and death (the classical Weil's disease). Leptospirosis onsets suddenly with headache, fever (typically to 102^0F, 39^0C), myalgia, conjunctival suffusion, and sometimes a transient rash. After that, the illness may be mild and self-limiting or severe and fatal (Adler and de la Pena Moctezuma 2010, Levett 2001, Ko et al. 2009).

1.5 INCIDENCE TRENDS OF LEPTOSPIROSIS

Though leptospirosis has been recognized for decades and considered a major public health problem, the paucity of literature on disease burden and varied clinical manifestations has made it a highly underreported disease. Multiple epidemics have been reported due to natural disasters and poor sanitary conditions in developing countries (Victoriano et al. 2009, Park 2011, Holla et al. 2018). Data is very limited regarding the true incidence of leptospirosis.

However, it is estimated that ≥10 per 100,000 people are affected with leptospirosis each year in tropical climates. In case of an epidemic, the cases can rise upto ≥100 per 100,000 people (Victoriano et al. 2009).

1.5.1 Global scenario of leptospirosis

It is estimated that there are 1.03 million leptospirosis cases and approximately 60,000 deaths each year, based on a systematic review of 80 published morbidity and mortality studies and databases from 34 countries with annual morbidity of 14.8 per 100,000 population (Costa et al. 2015). The disease's prevalence is often endemic at varying levels, from very low incidence in temperate regions (Rood et al. 2017) to hyperendemic with strong seasonality in tropical regions (Sumi et al. 2017). These estimates put leptospirosis as a leading zoonotic cause of morbidity and mortality. Moreover, morbidity and mortality were most prevalent in the world's poorest regions and areas where surveillance is not routinely performed. However, it is likely that those figures still underestimate the burden because leptospirosis patients are commonly misdiagnosed with dengue, malaria and other diseases.

The disease has been endemic in East and South-east Asia, Australia, Oceania, and South America (Zhang et al. 2012, Miraglia et al. 2013, Smith et al. 2013). Nevertheless, in recent years, occasional cases and outbreaks of human leptospirosis have been frequently reported from many European countries, North America and Africa, where leptospirosis is now being considered as an emerging or re-emerging infectious disease (Biggs et al. 2013, Goris et al. 2013, Traxler et al. 2014). Estimates of Disability Adjusted Life Years (DALYs) suggest that globally approximately 2.90 million DALYs are lost per annum from the approximately annual 1.03 million cases. Males are predominantly affected with an estimated 2.33 million DALYs or approximately 80% of the total burden. For comparison, this is over 70% of the global burden of cholera is estimated by the Global Burden of Disease 2010 study. Tropical regions of South and South-east Asia, Western Pacific, Central, and South America, and Africa had the highest estimated leptospirosis disease burden (Torgerson et al. 2015).

Table 1. 1 Comparison showing the global burden of leptospirosis with the top seven listed neglected tropical diseases

Diseases	Number of Cases	Deaths	DALYs (millions)
Intestinal nematodes	1,723 million	2,700	5.19
Leishmaniasis	10 million	51,600	3.32
Schistosomiasis	252 million	11,700	3.31
Leptospirosis*	1 million	**58,900**	2.90
Lymphatic filariasis	36 million	-	2.78

Food-borne trematodiasis	16 million	-	1.88
Rabies	1,100	26,400	1.46
Dengue	179,000**	14,700	0.83

*(Source: Torgerson et al. 2014 PLoS Negl Trop Dis 9(10): e0004122). *Is not a listed neglected tropical disease (severe cases only), **Incident (acute) symptomatic cases only.*

1.5.2 Indian scenario of leptospirosis

In India, leptospirosis is a major health problem related to the monsoons and poor sanitary conditions, with multiple epidemics reported in recent years (Pappas et al. 2008). India is endemic to leptospirosis since the early 20th century. Most outbreaks of leptospirosis in India are reported from the coastal regions of Gujarat, Maharashtra, West Bengal, Orissa, Kerala, Tamil Nadu, Karnataka, and the Andaman Islands. The highest incidence occurs during October to November, which coincides with the monsoon season in these parts (Himani et al. 2013).

The incidence of leptospirosis in developing countries is 10 – 100 per 100,000 people per year. By this estimate, India should report 0.1 - 1.0 million cases per year, but less than 10,000 cases are reported. However, only four states (Kerala, Gujarat, Tamil Nadu, and Maharashtra) report more than 500 cases per year. Andaman and Nicobar Islands, Andhra Pradesh, Assam, Goa, Delhi, Karnataka, Orissa, Puducherry, and Uttar Pradesh also reported cases. Kerala has reported leptospirosis cases from all districts, and this disease is the leading cause of mortality among infectious diseases prevalent in the state (Kumar 2013, Shivakumar 2008). Gujarat has reported cases from the southern districts of Surat, Valsad, and Navasari. Metropolitan cities like Chennai and Mumbai have also reported leptospirosis (Supe et al. 2018, Ramakrishnan et al. 2003). Recently, West Bengal, Punjab, Haryana, and Himachal Pradesh have also reported leptospirosis cases (Sethi et al. 2010, Goswami et al. 2014, DebMandal et al. 2011). The incidence of leptospirosis is 50 – 65 cases per 100,000 people per year in Andaman and Nicobar Islands (data from symptomatic patients) (Shivakumar 2008). The prevalence rate of leptospirosis reported is 38.1% from Calicut, 52.7% from Andaman and Nicobar Islands, and 32.9% from Chennai (data from asymptomatic patients during sero surveys) (Shivakumar 2008). More than 100 deaths per year of leptospirosis are reported from Gujarat, Kerala, and Maharashtra (Shivakumar 2014). Leptospirosis is grossly underreported in India primarily due to lack of awareness of the disease and the non-availability of diagnostic tests. A disease for which a lot is still to be learned on its pathogenesis and treatment principles, awareness of disease epidemiology and incidence patterns may allow for better prevention and treatment practices, especially in India (Bharti et al. 2003).

1.6 AVAILABLE TREATMENT OF LEPTOSPIROSIS

Treatment of leptospirosis varies with the severity and duration of symptoms at the time of clinical examination. Patients with mild, flu-like symptoms require only symptomatic treatment but are advised to seek medical help if they develop jaundice. Patients with severe leptospirosis require hospital admission and close observation (Levett 2004). Early treatment has been shown to offer the best clinical outcomes; results from controlled studies of treatment during the immune phase have yielded mixed results (Watt et al. 1988).

Mild leptospirosis is treated with doxycycline, ampicillin, azithromycin, or amoxicillin (Griffith et al. 2006). For severe leptospirosis, intravenous penicillin G has long been the drug of choice, although the third-generation cephalosporins, cefotaxime, and ceftriaxone have become widely used. Among a variety of antibiotics used, penicillins and cephems had the lowest minimum inhibitory concentration against leptospires. However, fundamental studies *in vitro* and *in vivo* reveal that streptomycin was the most effective anti-leptospiral antibiotic (Koyabashi 2001). Effective alternatives to streptomycin include gentamicin, tobramycin, and isepamicin antibiotics. When penicillins, cephems, tetracyclines, and macrolides with inadequate bactericidal activity are used to treat the disease, long-term therapy with sufficiently large doses may be required from an early stage of the disease until the appearance of antibodies (Kobayashi 2001). Several other antibiotics may be useful—for example, broth microdilution testing has shown sensitivity to macrolides, fluoroquinolones, and carbapenems (Murray and Hospenthal 2004)- but clinical experience with these agents is limited. Fluoroquinolone antimicrobials are another option for treating leptospirosis, although adequate human trials are lacking to support their use (Griffith et al. 2006).

Severe cases of leptospirosis can affect any organ system and can lead to multiorgan failure. Supportive therapy and careful management of renal, hepatic, hematologic, and central nervous system complications are important.

Porcine, bovine and canine vaccines are available against this organism (Levett, 2001). Human vaccines are available in some countries that contain killed spirochetes of the most prevalent serovar of that region. However, they present many disadvantages, including side effects, short duration of efficacy, and insufficient protection against other serovars. Thus antibiotics are the only treatment available. However, continuous usage may lead to antibiotic resistance in the pathogenic strains making it difficult to treat by the traditional methods. Thus it is important to look into alternative approaches that can potentially combat the infection in the near future. Though a wide range of antibiotics can be used to treat leptospirosis, and not a single clinical isolate showing resistance to antibiotics has been described so far. However, leptospirosis is a

re-emerging infectious disease (Dong et al. 2017) with limited understanding of pathogenesis. Like any pathogenic bacteria, infective *Leptospira* isolates might get antibiotic-resistant in near times, and a preliminary study to understand novel drug targets inside the bacterial cell (either by inhibition or activation) can further promote understanding the disease pathogenesis.

1.7 ALTERNATIVE APPROACH TO TREAT BACTERIAL INFECTION

1.7.1 Targeting bacterial ClpPs

Vaccination is the most viable strategy for controlling any infectious diseases, including leptospirosis. However, there is currently no available vaccine against leptospirosis for human use worldwide except for select countries such as France, Cuba, and Japan that have approved vaccines for risk populations (Teixeira et al. 2019). Owing to the large variability in local leptospiral strains in different countries or regions, the development of a universal vaccine is challenging. To date, prevention and treatment for leptospirosis in humans have mainly relied on conventional antibiotics. Overexploitation of conventional antibiotics eventually leads to developing a resistant form of microbes; therefore, alternatives are being investigated, including novel antibiotics, antibodies, probiotics, bacteriophages, and antimicrobial peptides (Ghosh et al. 2019). Anti-virulence approaches, phage therapy, and therapeutic antibodies are strategies that may yield drugs with high specificity and narrow spectra (Fernebro 2011). In this context, caseinolytic proteases P (ClpP) have garnered considerable attention as targets for antibacterial action after their direct relationship with bacterial virulence was proven in Gram-positive and Gram-negative bacteria (Mei et al. 1997, Gaillot et al. 2000, Hensel et al. 1995) in the late 90s. Bacterial Clps are an important class of proteases required for cellular homeostasis and protein turnover, to maintain vital cellular functions, particularly under stress conditions (Sauer and Baker 2011). Other than their role in maintaining protein quality, their ability to control key regulatory proteins' proteolysis aids in several developmental processes like cell motility, genetic competence, cell differentiation, sporulation, and important aspects of virulence. Due to their importance in many physiological processes and their conservation among diverse bacterial species, including human pathogens, it has led to a potential antibacterial target (Brötz-Oesterhelt and Sass 2014, Moreno-Cinos et al. 2019).

The modulation of ClpP proceeded from a factor isolated in the fermentation broth of *Streptomyces hawaiiensis*, which can deregulate the ClpP function (Brotz-Oesterhelt et al. 2005). The original group of eight factors discovered by Eli Lily and Company was reported to have antibacterial activity against *Staphylococcus* and *Streptococcus* strains (Michel and Kastner 1985). However, no suggested mechanism of action of the novel class of antibiotics

was described until 2005. The original cyclic acyldepsipeptides (ADEP1) structures from Eli Lily were characterized where ClpP was identified as their target (Brotz-Oesterhelt et al. 2005). Interestingly, ADEPs neither inhibit ClpP proteolytic activity nor interact with the catalytic sites; instead, these cyclopeptides bring about conformational changes in ClpP (Lee et al. 2010, Li et al. 2010). The active ClpP turns into unregulated proteolytic machinery from a highly regulated one, thus degrading proteins necessary for bacterial survival leading to bacterial death (Brotz-Oesterhelt et al. 2005).

The first natural ADEPs (ADEP1 and Enopeptin A) (Brotz-Oesterhelt et al. 2005, Osada et al. 1991) was improved by chemically synthesizing the improved congeners by derivatization program (Brotz-Oesterhelt et al. 2005). The synthesized ADEP2 and ADEP4 (**Figure 1.4**) had an 160-fold increased antibacterial activity and a boost in bioavailability and chemical stability against Gram-positive pathogens *S. aureus, S. pneumoniae*, and *E. faecalis* when treated *in vivo* in mice (Brotz-Oesterhelt et al. 2005, Hinzen et al. 2006). ADEP4 was able to eradicate *M. tuberculosis* by preventive binding between the cognate MycClpX and the MycClpP1P2 (Famulla et al. 2016). Further improvements in the ADEP1 structure led to ADEP10c and ADEP1g (**Figure 1.4**) with improved antibacterial activity (Socha et al. 2010, Carney et al. 2014). However, the activity of ADEPs were limited to Gram-positive pathogens until 2016 (Goodreid et al. 2016). A group of compounds was reported, ADEP26 (**Figure 1.4**), which not only killed Gram-positive *S. aureus, E. faecalis, S. pneumoniae, B. subtilis,* and *L. innocua*; but also displayed antibacterial activity against Gram-negative *Neisseria meningitidis* and *N. gonorrhoeae* (Goodreid et al. 2016). Besides ADEP antibiotics, several compounds have been identified with a similar mode of action against Clp proteases. Five clusters of ACP (activators of self-compartmentalizing proteases) were identified, among which ACP1b (**Figure 1.4**) with the most developed structure had significant bactericidal activity against both Gram-positive *S. pneumoniae, S. aureus* , and Gram-negative *N. gonorrhoeae, N. meningitidis, P. aeruginosa, L. monocytogenes,* and *Haemophilus influenza* (Leung et al. 2011). Further development of ACPs by derivatization program led to improved ACP1 analogs that were effective against *Neisserial* ClpP proteases ((Binepal et al. 2020). Another non-petide based natural activator of ClpP, sclerotiamide, has been recently identified, though the activity was only restricted to EcoClpP (Lavey et al. 2016). Recently, a species-selective activator for human ClpP, D9 (**Figure 1.4**) has been reported that binds to a unique aromatic amino acid network in the hClpP groove (Stahl et al. 2018).

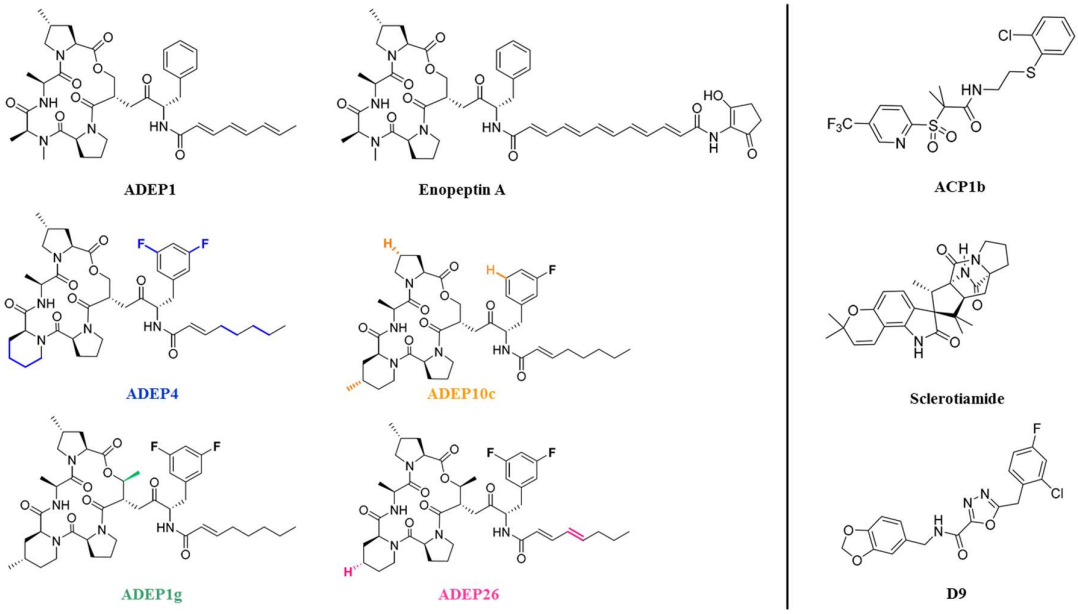

Figure 1. 4 Chemical structures of ClpP activators.
On the left, chemical structures of the mentioned ADEPs are shown. In the color matching their name, consecutive modifications respect to the previous ADEP are highlighted: the original ADEP1 and EnopeptinA (black), ADEP4 (blue), ADEP10c (orange), ADEP1g (green) , and ADEP26 (pink). On the right side, alternative structures extracted from screening : ACP1b, Sclerotiamide, and D9. (Source: Moreno-Cinos et al. 2019)

The other families of Clp protease modulators target the Clp-ATPases/unfoldases. Large cyclic peptides such as cyclomarine A (Schmitt et al. 2011), lassomycin (Gavrish et al. 2014), ecumicin (Gao et al. 2015), and rufomycin analog, RUF-I (Choules et al. 2019) (**Figure 1.5**) specifically target MycClpC1. They have shown their activity even against multi-drug resistant *Mtb* strains (Moreno-Cinos et al. 2019). Another example of ATP-unfoldase targeting compound is dihydrothiazpine334 (**Figure 1.5**) (Fetzer et al. 2017). This compound interacts with S. aureus ClpX (StaClpX) and provokes its deoligomerization. The virulence and the toxin production level of multi-drug resistant *S. aureus* strains were attenuated upon treatment with this drug mentioned above (Fetzer et al. 2017).

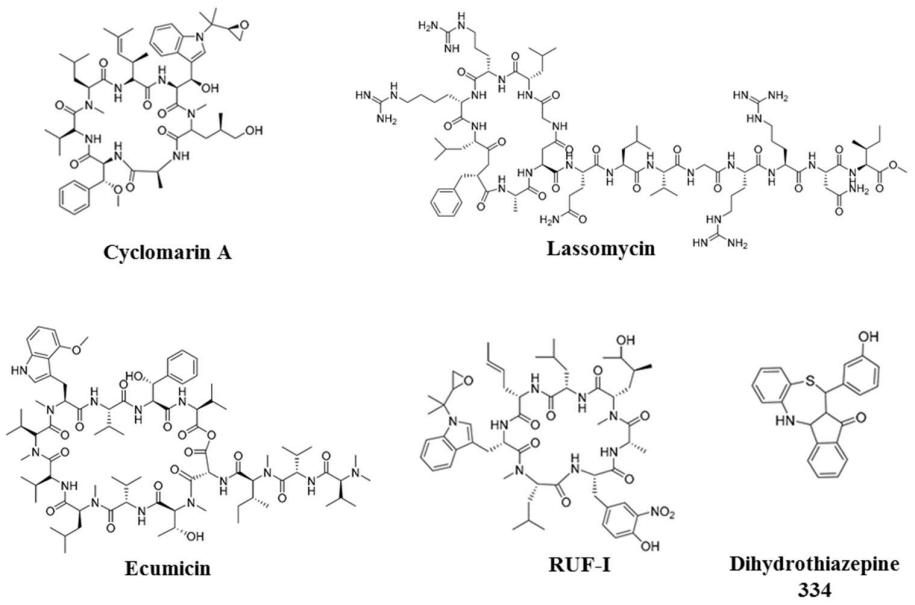

Figure 1. 5 Chemical structures of compounds targeting Clp-ATPases
(Source: Moreno-Cinos et al. 2019)

Apart from ClpP activation, novel class of ClpP inhibitors has also been reported with novel mechanisms (**Figure 1.6**). Covalent inhibition of the catalytic sites of ClpP by a class of small molecule inhibitors β-lactones is investigated in the context of virulence inhibition against *S. aureus* and *L. monocytogenes* (Böttcher and Sieber 2009, Böttcher and Sieber 2008). The optimized β-lactone molecule U1 have displayed inhibition against *P. falciparum* and *M. tuberculosis* (Compton et al. 2013, Rathore et al. 2010). Other identified ClpP inhibitors include phenyl esters (Hackl et al. 2015), heterocycles, pyrazolopyridine, and 2-(thiopen-2-yl) oxazole moieties (Pahl et al. 2015). A new activation mechanism has been reported by Gersch et al. when they found β-sultams (RKS07) to modify StaClpP catalytic serine selectively (Gersch et al. 2014). The alteration of catalytic residue triggers the disassembly of the active ClpP tetradecamer into two inactive heptameric rings (Gersch et al. 2014). *M. tuberculosis* ClpP1P2 (MycClpP1P2) reacts with known standard serine protease inhibitors such as chloromethyl ketones (Z-LY-CMK), which modify the serine and histidine residues of the active sites (Akopian et al. 2012). Peptide boronates have also been shown to directly interact with MycClpP1P2 active site causing inhibition and thereby preventing mycobacterial growth (Vahidi et al. 2020). Cediranib, an anti-cancer drug, was also proposed as a novel non-covalent inhibitor of MycClpP1P2 (Yang et al. 2019). Bortezomib, a known 26S proteasome inhibitor used in cancer treatment, also reported inhibiting MycClpP1P2 (Moreira et al. 2015). Substrate-based boronates (N-(picolinoyl)-Trp-Lys-boroMet), by Akopian et al. also displayed selective

targeting of MycClpP1P2 that induced growth inhibition (Akopian et al. 2015). New pyrrole cores,13i, identified as MycClpP1P2 inhibitors, exhibited antibacterial properties (Liu et al. 2018). Inhibition of *P. falciparum* growth and apicoplast segregation leading to parasite death was achieved by pyrimidine 33- a novel class of ClpP inhibitors (Mundra et al. 2017). Although, ClpPs of several organisms have been targeted, the inhibiton of Gram-negative bacterial ClpP remains very limited. In this context a new class of inhibitors with an α-amino diarylphosphonate warhead was reported that could inhibit *EcoClpP*. This family represents the irreversible inhibitors of serine proteases (Moreno-Cinos et al. 2018). One of the 14 identfied inhibitors, DPP 85, among the screened 150 compounds, was able to delay and decrease the growth of *E. coli* under nitric oxide stree (Moreno-Cinos et al. 2018). The result was consistent when *ΔclpP* mutant strain displayed impaired growth under the nitric oxide conditions (Robinson and Brynildsen 2015). Furthermore, administration of DPP 85 to the ΔclpP strain did not produce an additional effect, suggesting a ClpP mediated effect (Moreno-Cinos et al. 2018). Both antibacterial mechanisms of ClpP activation and inhibition comprise innovative approaches with the potential to control infections caused by multi-resistant bacterial pathogens due to the lack of cross-resistance to established antibiotic classes.

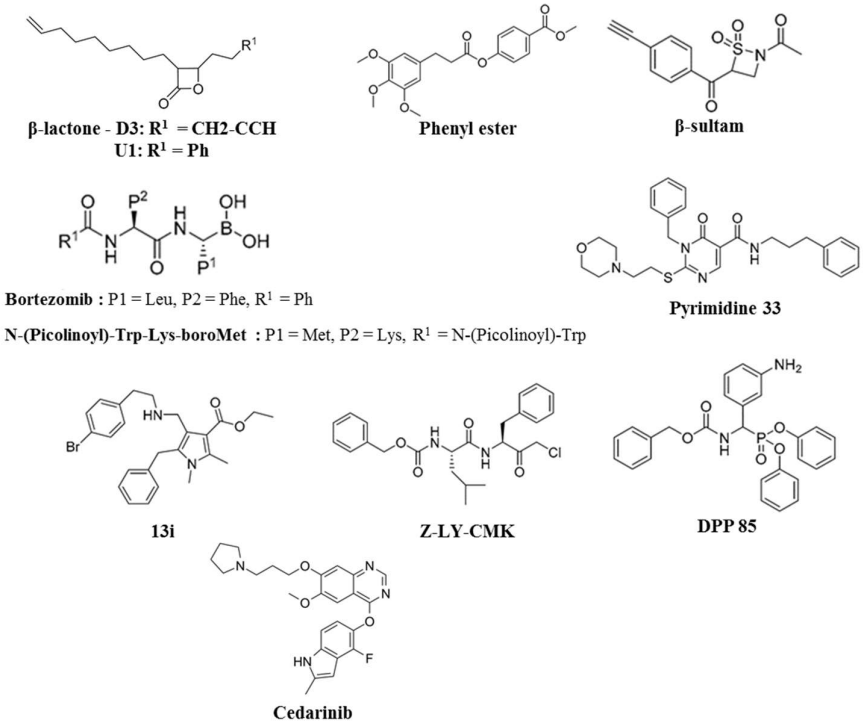

Figure 1. 6 Chemical structures of ClpP inhibitors.
(Source: Moreno-Cinos et al. 2019)

Bioinformatic analyses of the genomes of the sequenced strains of *Leptospira* show the existence of the *clp* system (Nascimento et al. 2004, Bulach et al. 2006, Picardeau et al. 2008, Ren et al. 2003) which might play an important role in the virulence and survival of *L. interrogans* in diverse hosts and environments. The clp system genes are conserved in both pathogenic and saprophytic species of *Leptospira* and constitute part of core genes (Picardeau et al. 2008). Although much is left to be explored about *Leptospira clp* system, inactivation of *clpB* and *HslUV* genes in pathogenic *Leptospira* showed reduced virulence and resistance to stress conditions in the absence of that particular gene (Lourdault et al. 2011, Dong et al. 2017). Considering the significance of *clp* genes, the study of the system can help understand the pathophysiology of *L. interrogans.* Therefore, proper insight into its molecular mechanism might pave the way for developing novel and selective, either inhibitory or activating agents (Böttcher and Sieber 2008, Brotz-Oesterhelt et al. 2005, Lee et al. 2010). The importance of caseinolytic proteases (Clps) in diverse species and the reason to consider it as an antibacterial target has been elucidated in the following sections.

1.8 PROTEOLYSIS IN BACTERIA

Energy-dependent proteolysis is a rudimentary process in bacteria where damaged and misfolded proteins are eliminated by cellular machinery to prevent detrimental aggregation (Sauer et al. 2004). Five distinct classes of proteases, namely ClpAP, ClpXP, FtsH, Lon, and HsIUV, carry out regulated proteolysis in Gram-negative bacteria (Langklotz et al. 2012), and additional proteases ClpEP and ClpCP are also found in several Gram-positive bacteria (Gur et al. 2011) (**Figure 1.7**).

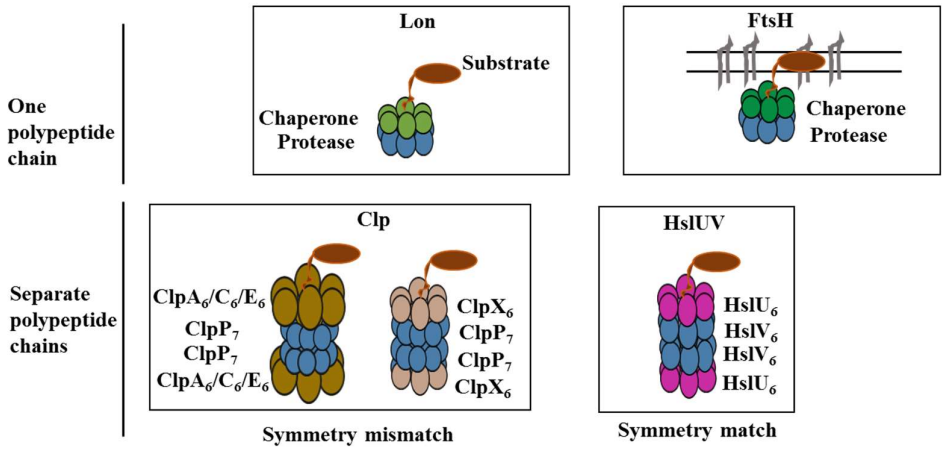

Figure 1. 7 Architecture of bacterial chaperone-protease complexes.
Cartoon depiction of the architecture of bacterial chaperone-protease-complexes. The protease core is always shown in blue, and the chaperone components in colors. The substrate (brown) is translocated through the axial pore and degraded inside the proteolytic chamber. The Lon and FtsH protease and chaperone subunits are encoded on one polypetide chain and they form symmetric hexameric assemblies. The Lon N-terminal domains can interact to form a double complex. FtsH features transmembrane segments that anchor it in the cell membrane (grey). In the Clp system, HslUV and the Proteasome system, chaperone and protease subunits are encoded on separate polypeptide chains. HslUV forms a symmetric particle with hexameric protease ($HslV_6$) rings associating with hexameric chaperone rings ($HslU_6$). The Clp architecture is characterized by a symmetry mismatch where heptameric protease particles ($ClpP_7$ subunits) associate with hexameric chaperone rings (ClpA/C/E_6, $ClpX_6$).

Caseinolytic protease P (ClpP) is highly conserved in prokaryotes and carries out 80% of the cellular protein degradation among these proteases (Maurizi et al. 1994). ClpP degrades various substrates, including proteins associated with cell division, motility, translation, and transcription (Feng et al. 2013, Flynn et al. 2003). ClpP is also an essential regulator of cellular growth, virulence, tolerance under heat shock, antimicrobial tolerance, and biofilm formation in different organisms, including pathogens like *Staphylococcus aureus*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *L. interrogans,* and *Enterococcus faecelis* (Nair et al. 1999, Frees et al. 2003, Frees et al. 2005, Lourdault et al. 2011, Dong et al. 2017, Zheng et al. 2020, Raju et al. 2014).

1.9 FEATURES OF CASEINOLYTIC PROTEASE (CLP)

1.9.1 Architectural structure of ClpP

ClpP is a highly conserved, multimeric serine protease discovered initially and extensively characterized in *Escherichia coli* (Akopian et al. 2012). ClpP homologs are widely distributed in bacteria and mitochondria and chloroplasts of eukaryotes as well. The crystal structure of several ClpP proteins like those of *E. coli* (Wang et al. 1997)*, Streptococcus pneumoniae*

(Gribun et al. 2005), *L. monocytogenes* (Dahmen et al. 2015), *S. aureus* (Geiger et al. 2011), *M. tuberculosis* (Li et al. 2016) reveals highly conserved architectural features (**Figure 1.8**).

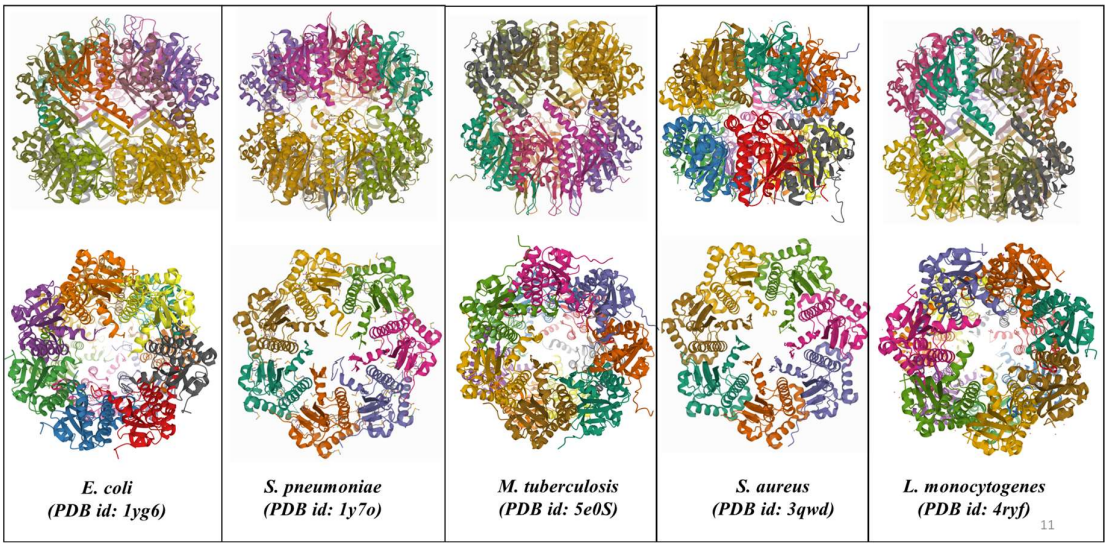

Figure 1. 8 Side and top views of the X-ray structures of tetradecameric ClpPs from *E. coli, S.pneumoniae, M. tuberculosis, S. aureus*, and *L. monocytogenes*.
The PDB ids of the respective structures are mentioned below each of X-ray structure.

Fourteen monomers of ClpP oligomerize to form two heptameric rings that stack back to back as a hollow cylinder of tetradecameric peptidase with 14 proteolytic active sites enclosed within its central chamber (Wang et al. 1997) (**Figure 1.9 A**). The axial pore of each ring (~10 Å diameter) acts as the entry point to the proteolytic chamber's interior (**Figure 1.9 B**). The ClpP structure shows three distinct features: N-terminal loops, a large head domain, and a handle domain (**Figure 1.9 C**). The N-terminal loops protrude on the cylinders' axial sides and facilitate the interaction with assisting chaperone proteins (Joshi et al. 2004). The head domain or the globular region comprises the active site residues in the barrel's inner chamber and highly hydrophobic surfaces responsible for the intra-ring subunit interface (Lee et al. 2010), and the handle domain assists in interaction with the counterpart on the opposite ring (Alexopoulos et al. 2012).

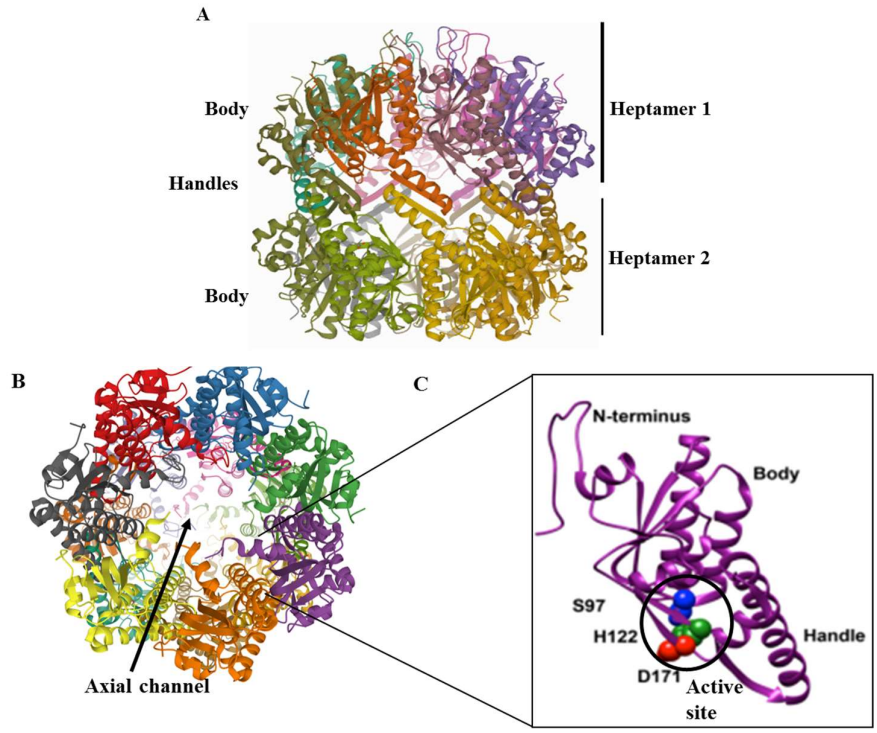

Figure 1. 9 Structure of the full wild type *E. coli* ClpP tetradecamer (A: side view, B: top view) and the ClpP monomer (C).
Every monomer is shown in a different color in cartoon representation. The monomer is show to emphasize the active site and the catalytic triad (S97, H122, D171) is shown in ball representation (PDB ID: 1yg6) [Conceptualised and redrawn from (Wang et al. 1997)].

The ClpP tetradecamer in *E. coli* consists of 14 identical subunits/protomers. Nevertheless, it can be found as a homomeric or heteromeric assembly where different isoforms of ClpP are present. In *M. tuberculosis* and *Chlamydia trachomatis,* ClpP1P2 is a heteromeric tetradecamer where each ring is formed with 7 protomers of different isoforms (Akopian et al. 2012, Pan et al. 2019). Examples of heteromeric assembly include cyanobacteria mixing ClpP and ClpR, an inactive variant of ClpP, leading to various tetradecameric complexes (Nishimura and van Wijk 2015). *Pseudomonas aeruginosa* and *Clostridium difficile* also harbor ClpP1 and ClpP2 isoforms; however, they only form separate tetradecamers that perform distinct functions in the cell (Hall et al. 2016, Lavey et al. 2018).

1.9.2 Activation of ClpP by ATPases

In *E.coli,* where the biochemical properties of EcoClpP have been extensively studied, the peptidase ClpP has its active sites for peptide bond cleavage inside its proteolytic chamber accessible only through the narrow axial pores of N-terminal, and the entry restricted to unfolded polypeptides only (Wang et al. 1997). By itself, the EcoClpP can hydrolyze small peptides upto six amino acid residues but not longer peptides and proteins (Woo et al. 1989,

Thompson and Maurizi 1994). To degrade large globular proteins, it requires the presence of an AAA+/ATPase chaperone such as EcoClpX, EcoClpA, or ClpC in other species (Kress et al. 2009). The Clp chaperones are highly conserved in prokaryotes and are classified into two main classes. Class I members include ClpA, ClpB, ClpC, ClpD, ClpE, and ClpL, contain two ATP nucleotide-binding domains or AAA+ (ATPases associated with various cellular activities) domain that have Walker A and Walker B nucleotide-binding motifs. Proteins of Class II include ClpX and ClpY, which are smaller and contain only 1 AAA+ domain (Sauer and Baker 2011).

The ATPase complex, a ring-shaped hexamer, aligns co-axially with the homomeric EcoClpP tetradecamer to form the active 4-ring ATP-dependent protease /AAA+ protease (Maurizi 1992, Maurizi et al. 1998, Kim et al. 2001). In a heteromeric assembly of *L. monocytogenes* ClpP1P2 tetradecamer (LisClpP1P2), it has been observed that the LisClpX ATPase complex binds to the hydrophobic pockets of the N-terminal domain of ClpP2 protomers forming a 3-ring AAA+ protease (Gatsogiannis et al. 2019, Pan et al. 2019, Leodolter et al. 2015). These complexes selectively recognize the polypeptide substrates and provide the energy required for protein unfolding and translocate the linear polypeptide into the ClpP proteolytic chamber for degradation through ATP hydrolysis (Alexopoulos et al. 2012).

1.10 IMPORTANCE OF CASEINOLYTIC PROTEASES

One of the most important functions of Clp system is protein stress management,i.e., in situations when mistranslated, misfolded, or aggregated proteins accumulate in the bacterial cell as a result of, e.g., heat stress or antibiotic interference with the ribosomal machinery (Flynn et al. 2003). **Figure 1.10** outlines the process of protein degradation by the ClpP and chaperone complex. In a first attempt, Clp-ATPase goes for the refolding process of irregular proteins in an ATP-dependent manner, independently of ClpP. If refolding is unsuccessful, Clp-ATPases directs the protein to the proteolytic core ClpP, where it is unfolded and degraded, as shown in **Figure 1.10**.

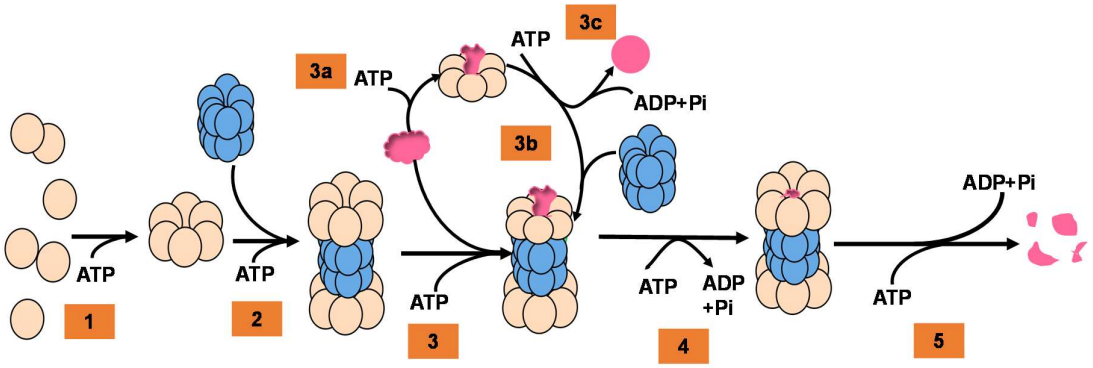

Figure 1. 10 General mechanism of ATP-Dependent protein degradation.
A model for the ATP-dependent degradation pathway is shown for a generic Clp-like protease, composed of a Clp ATPase component (shown in light orange) and a proteolytic component (shown in blue). The substrate is shown in pink. The alternate pathway of protein remodelling by the ATPase component is also shown (Step 3a-c). [Conceptualised and redrawn from (Gottesman et al. 1993)]

In every species, a number of chaperone-protease complexes coexist. The significance of each of them in every organism is determined by the importance of their role and overlap of their functions.

The assembly of the ATPases and protease components is indispensable for the regulated degradation of the substrates. One way to ensure that only fully synthesized proteins are circulated inside the cell is by having incompletely translated proteins removed by the Clp system (Flynn et al. 2001). A range of unfolded or partially unfolded proteins with low amounts of tertiary structure and/or a high amount of exposed hydrophobic stretches is recognized and degraded, such as the protease's model substrate casein (Raju et al. 2012). The ATPases recognize, unfold, and introduce these damaged proteins inside the proteolytic chamber to ensure protein quality control inside the cell (Olivares et al. 2016, Konovalova et al. 2014). With the common presence of diverse ATPases/unfoldases, the ClpP activity is tightly regulated, as substrate selection is dependent on a specific unfoldase (Gur et al. 2013). Inherent or added sequence tags often perform this target identification at the N- or C-terminus of these substrates (Flynn et al. 2003). For instance, when *E. co*li ribosomes stall during translation due to mRNA truncation or tRNA depletion, a rescue transfer-messenger RNA (tmRNA) or ssrA-ribosome rescue system resolves the block by mediating the addition of an 11 amino acid sequence (AANDENYALAA) at the C-terminal of the nascent polypeptide (Tu et al. 1995, Keiler et al. 1996, Karzai et al. 1999). This peptide sequence called the ssrA-tag targets the modified protein for degradation (Keiler et al. 1996). The degradation of ssrA-tagged proteins is a central feature of protein quality control in all bacteria by preventing the accumulation of misfolded and unfinished proteins inside the cell (Farrell et al. 2005). Studies have shown that

EcoClpXP, EcoClpAP, and EcoFtSH proteases are all capable of degrading ssrA-tagged proteins in an ATP-dependent manner (Gottesman et al. 1998, Herman et al. 1998). The Clp-adaptors binding to Clp-ATPases upon signals or stresses influences the substrate choice of chaperone-protease complex, thereby further tightening the proteolysis regulation. SspB, RssB, and UmuD for EcoClpX (Flynn et al. 2004, Neher et al. 2003, Peterson et al. 2012), EcoClpS for EcoClpA or MecA for *B. subtilis* ClpP (BacClpP) (Flynn et al. 2001, Kirstein et al. 2006) are some examples of these Clp adaptors. Recent identification of small anti-adaptor proteins regulating the activity of the adaptors provides an additional layer of complexity for the control and fine-tuning of ClpP protease. (Battesti et al. 2013)

Clp proteases also control crucial developmental processes in bacteria via proteolysis of regulatory key elements such as transcription factors. In *Bacillus subtilis,* the regulation of motility, exoenzyme synthesis, spore formation, and genetic competence is driven by Clp (Msadek et al. 1998, Pummi et al. 2002, Turgay et al. 1998). In *Caulobacter crescentus,* a Clp protease governs cell differentiation (Jenal and Fuchs 1998). In some Actinomycetales, including *M. tuberculosis*, *Corynebacterium glutamicum,* and *Streptomyces lividans*, ClpP is even essential for growth under moderate conditions *in vitro* (Engels et al. 2004, Gominet et al. 2011, Raju et al. 2012, Sassetti et al. 2003). Due to its prominent role in protein turnover and regulated proteolysis, inactivation or deletion of ClpP causes severe phenotypes and even growth inhibition under stress conditions in many bacterial species, including important human pathogens like *S. aureus*, *L. monocytogenes*, and *Salmonella typhimurium* (Mei et al. 1997, Hensel et al. 1995, Gaillot et al. 2000). Consequently, Clp proteases have attracted considerable attention in evaluating their potential as drug targets.

1.10.1 ClpP and Clp-ATPases as anti-virulence targets

In several bacterial species, ClpP or Clp-ATPases are crucial for developing bacterial virulence (Frees et al. 2003, Frees et al. 2007). In *Listeria monocytogenes*, a prominent cause of foodborne disease listeriosis, two LisClpP isoforms are expressed. LisClpP2 is essential for virulence, the production, and secretion of functional listeriolysin, an exotoxin required for intracellular growth of the pathogen in macrophages; the function of LmClpP1 is unknown. Inhibition of LisClpP2 by β-lactones led to decreased virulence in the food-borne pathogen (Balogh et al. 2017, Böttcher and Sieber 2009). A *clpP* deletion mutant in *L.monocytogenes* was avirulent in the mouse, even when applied at a 5000-fold higher infective dose than the wild-type (Gaillot et al. 2000). Studies also show that among the Clp-ATPases, LisClpC promotes early escape of *Listeria* from the phagosomal compartment in macrophages

(Rouquette et al. 1998), and LisClpE was also shown to contribute to virulence (Nair et al. 1999).

In *S. pneumoniae*, an important causative agent of lung infection, *clpP* deletion led to a significant virulence reduction in several mouse models (Robertson et al. 2002, Kwon et al. 2003, Kwon et al. 2004). In *E. faecelis*, deficiency of ClpP or Clp ATPases led to the loss of virulence in an invertebrate model (Cassenego et al. 2016)

Similarly, in *S. aureus*, the most frequent cause of nosocomial infection among Gram-positive bacteria, *clpX* and *clpP* deletion mutants were shown to down-regulate the transcription of genes encoding several secreted virulence proteins, among them -toxin, extracellular proteases, lipases, and different hydrolases (Frees et al. 2003, Michel et al. 2006) (Frees et al., 2003; Michel et al., 2006). In addition to the specific roles in regulating the abundance of the particular virulence factors mentioned above, it shall not be forgotten that Clp proteases help bacterial cells to bear diverse sorts of stress by their general activities in protein quality control and that the entry of bacterial pathogens into the hostile environment of the host imposes enormous stress on the bacteria.

In *S. typhimurium*, the deletion of *clpP* led to the mutant strain's growth hindrance in a mouse model (Hensel et al. 1995). More recent examples include the role of ClpP in the pathogenesis of *P. aeruginosa* and *Legionella pneumophila* (Zhao et al. 2016, Qiu et al. 2008).

In *M. tuberculosis*, the entire Clp protease system is essential for the species' growth, virulence, and viability (Ollinger et al. 2012, Raju et al. 2012, Sassetti et al. 2003). MycClpP1/P2 is activated by N-blocked dipeptides (Akopian et al. 2012) or benzoyl-leucine-leucine (Li et al. 2016), enhancing the proteolytic activity *in vitro*. These MycClpP1P2, together with chaperones MycClpX and MycClpC1, were essential for pathogen viability and infectivity *in vitro* (Raju et al. 2012).

In summary, the relevance of ClpP proteases in bacterial pathogenesis offers an untapped target for new antivirulence and antimicrobial drugs (Culp and Wright 2016).

1.10.2 ClpP and ClpP-ATPases as new targets for antibiotic attack

As reported in the literature, ClpP may serve as an anti-virulence target, inhibiting bacterial survival and spreading during host infection. However, in recent years ClpP has also been discovered to serve as an antibiotic target. It was found that new antibacterial acyldepsipeptides (designated “ADEPs”) take ClpP out of its physiological context to cause bacterial death (Brotz-Oesterhelt et al. 2005).

In the early 1980s, a group of eight closely related ADEPs was isolated from *Streptomyces hawaiiensi* NRRL 15010 and patented as the A54556 (factor A) complex provided initial data on their antibacterial action *in vitro* (Michel and Kastner 1985). Starting from factor A, which was renamed ADEP1 new, improved derivatives ADEP2 and ADEP4 were synthesized (Brotz-Oesterhelt et al. 2005). The new synthetic ADEP derivatives reached minimal inhibitory concentrations (MICs) in the sub- g/ml range for the three major pathogens among Gram-positive species- *S. aureus, S. pneumoniae,* and *E. faecium*, including multi-resistant clinical isolates, and in mouse models of bacteria infection, their efficacy even surpassed that of commercial competitive antibiotic linezolid (Brotz-Oesterhelt et al. 2005). Additionally, no cross-resistance with commercial antibiotics was observed, suggesting that ADEPs might interfere with a new bacterial target. This notion was further supported during the initial mode of action studies, which failed to identify the target in one of the classical metabolic pathways, including the syntheses of DNA, RNA, proteins, fatty acids, and peptidoglycan. The target was identified in the course of complementary genetic and biochemical approaches. The characterization of ADEP-resistant mutants revealed mutations in the *clpP* gene, and immobilized ADEP retained ClpP selectively during affinity chromatography (Brötz-Oesterhelt et al., 2005). Structural and biochemical analyses on the interaction of ADEPs with ClpP revealed that ADEPs deregulate ClpP in an unprecedented and multifaceted manner (Malik and Brötz-Oesterhelt 2017). *In vitro* studies using purified BacClpP and EcoClpP showed that in the absence of ClpP-ATPases, ADEPs induce the oligomerization of ClpP monomers into the tetradecameric complex which, however, is not a tightly regulated proteolytic machinery (Lee et al. 2010, Li et al. 2010). ADEPs prevent the interaction of BacClpP with its ClpATPases and thus inhibit the degradation of natural Clp protease substrates. Moreover, preformed BacClpP/ClpATPase complexes were even disassembled in the presence of ADEP (Kirstein et al. 2009). This way, the ADEPs uncouple the strictly regulatory proteolytic capacity of ClpP, thereby converting ClpP from a regulated to an uncontrolled protease that degrades fully or partly unfolded or flexible proteins like casein as well as nascent polypeptides at the ribosome independently of Clp-ATPases (**Figure 1.11**) (Brotz-Oesterhelt et al. 2005, Kirstein et al. 2009, Leung et al. 2011).

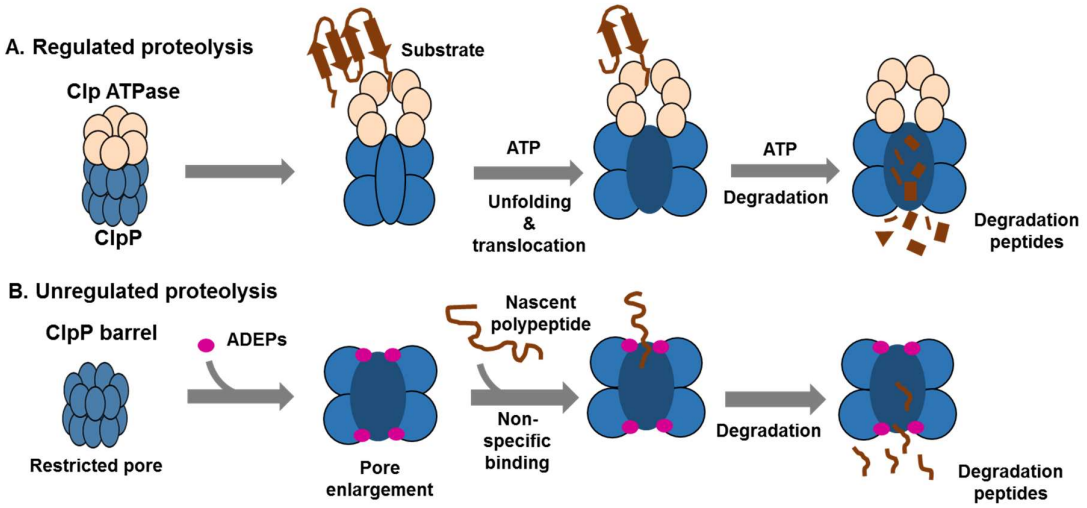

Figure 1. 11 ADEPs unleash the proteolytic activity of ClpP.
Model on the mode of action of ADEPs. ADEPs deregulate ClpP in a multi-layered fashion: ADEPs induce the oligomerization process of ClpP monomers into the tetradecameric complex and compete with Clp-ATPases for the same binding site on ClpP (**A and B**). As a result of ADEP binding, the interaction of ClpP with corresponding Clp-ATPases is prevented leading to the inhibition of the natural functions of Clp in protein turnover. Moreover, ADEPs uncouple the degradative capacity of ClpP from strict regulation by Clp-ATPases to permit the proteolysis of unstructured or flexible proteins or nascent polypeptides. [Conceptualised and redrawn from (Frees et al. 2013)].

Complementary X-ray crystallography studies yielded structures of ADEP in complex with BacClpP and EcoClpP, which provided a reason for the biochemical observations. The crystal structures revealed ADEP binding at the outer rim of the apical and distal surfaces of the ClpP tetradecamer in a 1:1 stoichiometry. Each ADEP molecule binds at the interface of two adjacent BacClpP subunits and establishes contacts to both, thereby stabilizing the oligomeric ring structure, which explains the observation that monomeric BacClpP is transferred to the tetradecameric state in the absence of BacClp-ATPases (Kirstein et al. 2009). Furthermore, the ADEP binding pocket at EcoClpP is precisely the previously suggested position as the contact point for the IGF/IGL loops of EcoClpX and EcoClpA (Kim et al. 2001). Thus, the cyclopeptides compete with the Clp ATPases' specific loop regions (IGF and IGL) that bind into the ClpP grooves in the apical region. By interaction with those grooves or pockets situated between the two adjacent monomers, ClpP tetradecameric complex adopts the active conformation of an ATP-dependent protease. The active conformation broadens the axial pores of the ClpP tetradecamer, but it lacks the selective and regulatory function of Clp-ATPase. ADEP binding induces major conformational changes in the N-terminal part of each ClpP subunit. The action triggers a closed to the open-gate structural transition of the ClpP N-terminal segments that enlarge the substrate entrance pore to the degradation chamber of the

ClpP tetradecameric complex, which now allows the entry of poorly structured regions of non-native protein substrates (Li et al. 2010, Lee et al. 2010). The observations were further corroborated by Gersh et al. 2015, where it was reported that in *S. aureus* Clp-ATPases and ADEPs activate the StaClpP protease by conformational changes. Considering this destructive capacity of ADEP-activated ClpP toward unstructured polypeptides or proteins, it appears that essential proteins in the bacterial cell are depleted in vital processes of the bacterial metabolism, thus already providing a plausible explanation for the inhibition of growth and bacterial killing. Though the ADEP-activated ClpP degrades more than one essential protein, studies reveal that at higher concentrations of ADEP, an essential cell division protein FtsZ is preferentially degraded. At low inhibitory ADEP levels close to the MIC, the syntheses of macromolecules including DNA, RNA, proteins, fatty acids, or peptidoglycan are not affected, and the bacteria are still able to produce biomass in considerable amounts. However, the cells are already unable to divide. At these ADEP concentrations, bacterial cell division is stalled due to the spatial mislocalization of central divisome proteins as FtsZ is reported to be particularly prone to degradation by the ADEP-ClpP complex (Sass et al. 2011). By preventing cell division, ADEP inhibits a vital cellular process of bacteria that is not targeted by any therapeutically applied antibiotic so far. Notably, by using this mechanism of action, ADEPs were the first antibiotics to cause bacterial death by over-activating rather than inhibiting their target. Their unique antibacterial potency makes them promising lead structures for future antibiotic development. These findings highlighting ClpP as a putative novel therapeutic target contributed to further studies of the evaluation of the ADEP class, i.e., by exploring the activity of structurally modified ADEP derivatives (Socha et al. 2010).

1.11 SIGNIFICANCE OF STUDYING CASEINOLYTIC PROTEASES OF PATHOGENIC LEPTOSPIRA

Although most organisms possess a single ClpP protein with conserved fold, the genomes of some microorganisms encode two or more ClpP isoforms. The ClpP of important pathogens like *L. monocytogenes*, *M. tuberculosis,* and *C. trachomatis* have been found to assemble into hetero-oligomeric complexes composed of two homoheptamers ClpP1 and ClpP2, for active functioning of the protease (Akopian et al. 2012, Geiger et al. 2011, Pan et al. 2019). The caseinolytic protease existing in one isoform in bacteria has been studied in detail, but the function and regulation of the ClpP system with more than one ClpP isoform are poorly understood. It has been shown that inhibition or activation of the ClpP1P2 complex can be a promising approach to combat disease pathogenesis (Famulla et al. 2016, Sass et al. 2011).

Bioinformatic analysis of the genome of the sequenced strains of *L. interrogans* shows the presence of *clpA, clpB, clpC, clpX,* two homologs of *clpP- clpP1* and *clpP2, clpS, clpY,* and *clpQ*. Studying the isoforms of *clpP* genes and essential regulatory ATPases, which might play an essential role in the virulence and survival of pathogenic *L. interrogans*, will help understand the physiological function of the *clpP* system and may also represent future therapeutic targets. As no work has been reported on *Leptospira* Clp system in India, characterizing the *clpPn*genes will be an important step in understanding the pathophysiology of *L.interrogans.* Moreover, recent studies on the *clpB* and *HslUV* (*clpYQ*) deletion mutants of *L. interrogans* showed reduced virulence and resistance to stress conditions than wild-type variants (Lourdault et al. 2011, Dong et al. 2017). Thus, it can be hypothesized that *the clpP system also play*s a role in maintaining virulence and cell homeostasis in *L. interrogans,* just as it does in other bacterial species. Taking the above into consideration, studying the role of ClpP1 and ClpP2 as a caseinolytic subunit and ClpX as an ATPase factor by generating recombinant protein will pave the way to develop new therapeutic measures against leptospirosis in the near future.

1.12 AIMS OF THE THESIS

1. Cloning, expression, and purification of the selected ClpP proteins- ClpP1, ClpP2, and ClpX of *L. interrogans* serovar Copenhageni Strain Fiocruz L1-130.
2. Molecular and biochemical characterization of the recombinant leptospiral Clps.
3. Understanding the role of ADEP– a new class of antibiotics in targeting ClpPs of *L. interrogans* under *in vitro* conditions.
4. Functional analysis of saprophytic *L. biflexa* using gene deletion by homologous recombination.

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Chapter 2

Insights to the assembly of functionally active leptospiral ClpP1P2 protease complex along with its ATPase chaperone ClpX[1]

2.1 ABSTRACT

The *L. interrogans* genome is predicted to encode multiple-isoforms of caseinolytic proteases (ClpP1 and ClpP2). The ClpP proteins, with the aid of its ATPase chaperone, are known to be involved in establishing cellular proteostasis and have emerged as a target for developing new antibiotics. We report the molecular characterization of recombinant LepClpP1 (LepClpP1) and LepClpP2 of *Leptospira* along with its ATPase chaperone LepClpX. The two isoforms of LepClpPs, when coupled together in equivalent concentration, exhibit optimum activity on small fluorogenic peptide substrates, whereas the pure LepClpP isoforms are enzymatically inactive. Isothermal Titration Calorimetry (ITC) analysis suggests that the two LepClpP isoforms bind each other moderately in a 1:1 stoichiometry with a dissociation constant of 2.02 ± 0.1 μM at 37ºC and is thermodynamically favored. Size exclusion chromatography (SEC) fractionate the majority of pure LepClpP1 at ≥ 308 kDa (14-21-mer) and the pure LepClpP2 at 308 kDa (tetradecamer) whereas, the functionally active LepClpP isoforms mixture fractionate as tetradecamer. The distinct and unprecedented oligomeric form of LepClpP1 was also evident through native-gel and dynamic light scattering (DLS). Moreover, the LepClpP isoforms mixture formed after the site-directed mutation of either or both the isoforms at one of the catalytic triad residues (Ser 98/97 to Ala 98/97) resulted in complete loss of protease activity. The LepClpP isoforms mixture gets stimulated to degrade the casein substrate in the presence of LepClpX and an energy-dependent manner. On the contrary, pure LepClpP1 or the LepClpP2 isoform associated with LepClpX are incapable of forming operative protease.

[1] This chapter is partly adapted from the published manuscript and reprinted with permission from Dhara, A.; Hussain, M. S.; Datta, D.; Kumar, M., "Insights to the Assembly of a Functionally Active Leptospiral ClpP1P2 Protease Complex along with Its ATPase Chaperone ClpX". ACS Omega 2019, 4, (7), 12880-12895. MK conceived and supervised the study; MK and AD designed experiments and analyzed data; AD performed experiments; MSH performed SEC analysis; DD performed and analyzed DLS experiment; MK, AD, and MSH wrote the manuscript.

The reported finding suggests that in *Leptospira*, the LepClpP protease complex's enzymatic activity in the presence or absence of co-chaperone is performed solely by the tetradecamer structure hypothesized to be composed of 2-stacked LepClpP heptameric rings, wherein each ring is a homo-oligomer of LepClpP1 and LepClpP2 subunits. Understanding the activities and regulation principle of multi-isoforms of ClpP in pathogenic bacteria may aid in intervening in disease outcomes, particularly to the co-evolving antibiotic resistance strains.

2.2 INTRODUCTION

L. interrogans is a spirochete of zoonotic importance responsible for causing leptospirosis disease in animals and humans worldwide (Bharti et al. 2003). It is estimated that over a million human cases of severe leptospirosis are reported each year globally, with approximately 60,000 deaths from this disease (Costa et al. 2015). It is worth noting that leptospirosis is a serious economic problem in developing tropical and subtropical countries (Victoriano et al. 2009, Torgerson et al. 2015). Each year, there are significant economic losses due to reproductive disorders in cattle, sheep, pigs, and horses suffering from leptospirosis (Ellis 2015). Despite the severity of leptospirosis and its global importance, the molecular mechanisms of the *Leptospira* pathogenesis are not well understood, possibly due to difficulty in studying the causal agent by reverse genetics approach (Picardeau 2015). Identifying the *Leptospira* virulence factors and characterization of their activity is particularly important for understanding the disease's mechanisms. In the last two decades, there is a growing list of pathogens shown to be impaired in their ability to infect or cause disease when lacking the components of the Clp protease system (Hensel et al. 1995, Mei et al. 1997, Frees et al. 2003a, Zhao et al. 2016, Gaillot et al. 2000, Kwon et al. 2004, Sassetti et al. 2003, Bhandari et al. 2018, Frees et al. 2003b) including the chaperone-peptidase complex (ClpY-Q) in pathogenic spirochete *L. interrogans* (Dong et al. 2017). Caseinolytic protease system is composed of the core catalytic components, regulatory chaperones (ATPases), and the adaptor proteins (Olivares et al. 2018). It has been observed that *E. coli* ClpP forms complexes with AAA+ (ATPases associated with various cellular activities) chaperones, ClpX, and ClpA (Gottesman et al. 1993, Maurizi et al. 1998). The Clp adaptors (Neher et al. 2003) and anti-adaptors (Battesti et al. 2013) provide other means for the extensive regulation of ClpP-dependent protein degradation. Clp adaptor protein binds to the N-terminal domain of ClpA, altering its substrate specificities (Kress et al. 2009).

The core catalytic components of the Clp protease system in leptospires are encoded by *clpP1, clpP2*, and *clpQ* gene. The protease complex of ClpP and it co-chaperone constitutes a

fascinating Clp protease system that is still required to be explored for designing suitable drug either by targeting protein activities or protein interactions during cellular proteostasis. One of the *Leptospira* caseinolytic protease complex ClpQ-ClpY (HslUV) (Lourdault et al. 2011) has been demonstrated to have a critical role in leptospiral survival in hosts and transmission of leptospirosis (Dong et al. 2017). However, there is a lack of biochemical and functional information regarding multi-ClpP isoforms present in the *Leptospira*.

Caseinolytic protease P (ClpP) is a well-conserved cylindrical/barrel-shaped serine protease in most bacterial species (Liu et al. 2014). A bacterium needs such protease to maintain protein homeostasis. Bacterial ClpP has an essential role in the degradation of misfolded or damaged proteins (Wickner et al. 1999), virulence (Gersch et al. 2012), numerous regulatory processes (Culp and Wright 2017, Frees et al. 2014, Viala and Mazodier 2003) such as cell division, stress tolerance, morphological differentiation, and antibiotic resistance. Unlike other serine proteases, ClpP proteases have evolved as self-compartmentalized barrel-shaped enzymes to establish cellular proteostasis (Gersch et al. 2016). Such a scaffold of ClpP prevents the bacterial cytosol matrix from breaching its catalytic site (Ser-His-Asp) harbored inside the barrel-shaped enzyme. In recent years, with the advent of ClpP-targeting anti-bacterial compounds, the primary emerging focus of such a study is to develop an alternative therapy for antibiotic-resistant and persistent microbes (Malik and Brötz-Oesterhelt 2017). While ClpPs from single isoform expressing bacteria has been studied in detail, the function and regulation of species, including *L. interrogans* with more than one *clpP* gene, are still poorly understood. Several pathogenic bacteria like *Mycobacterium tuberculosis*, *Listeria monocytogenes, Chlamydia trachomatis*, and *Pseudomonas aeruginos*a that harbor two isoforms of ClpP have been demonstrated to play an indispensable role in virulence (Gaillot et al. 2000, Hall et al. 2016, Ravikiran M. Raju et al. 2012, Wood et al. 2018). These multiple ClpP isoforms tend to reveal structural and functional disparity in an organism-specific manner. It has been established that in *E. coli* (Maurizi et al. 1990) and *Mycobacterium* (Akopian et al. 2012), the ClpP protomers must undergo proteolytic pre-processing at the N-terminal for achieving functional activity. Nevertheless, in many other pathogenic bacterial ClpP protomers (Zeiler et al. 2013, Hall et al. 2016, Wood et al. 2018) with multiple isoforms, the peptidase activity has been demonstrated even without such pre-processing. The barrel-shaped ClpP protease with axial pores strictly gives access to the unfolded polypeptide generated by ATP-dependent regulatory chaperones. Probing the behavior of ClpP systems in multi-isoform containing organisms anticipates further understanding of the function and regulation of ClpP systems leading to its clinical exploitation in intervening disease outcomes. A comprehensive

molecular characterization of multi-ClpP isoforms of *Leptospira* and its co-chaperone ClpX was executed in the present study.

2.3 RESULTS AND DISCUSSION

2.3.1 Caseinolytic protease genes in the *Leptospira* genome

The genome analysis of the sequenced spirochete, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 shows that it harbors various genes of caseinolytic protease (*clp*) system. The caseinolytic protease system in *Leptospira* is composed of genes encoding catalytic, regulatory, and adaptor proteins. The set of genes encoding catalytic components of the *clp* system in *Leptospira* are *clpP1* (*LIC11417*), *clpP2* (*LIC11951*), and *clpQ* (*LIC11600*). The Clp AAA+ (ATPases associated with various cellular activities) are encoded by *clpX* (*LIC11418*), *clpA* (*LIC11814*), *clpB* (*LIC12017*), *clpC* (*LIC10339*) and *clpY* (*LIC11601*) genes, whereas the adaptor proteins are encoded by *clpS* (*LIC11356* and *LIC11815*) (**Figure 2.1** and **Table 2.1**).

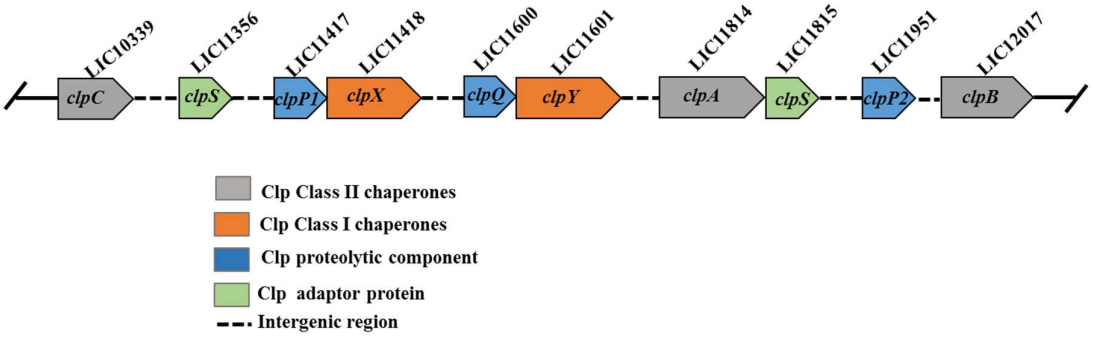


Figure 2. 1 A schematic arrangement of genes encoding caseinolytic protease (*clp*) system in *L. interrogans* genome.
The arrangement of *clp* genes and its annotation in *Leptospira* genome are represented in an arrow diagram (not scaled) based on bioinformatics analysis and previous report (Lourdault et al. 2011). Caseinolytic protease system in *Leptospira* is composed of genes encoding catalytic subunits (*clpP1, clpP2, clpQ*; blue color), Clp AAA+ subunits (*clpA*, *clpB, clpC, clpX*, and *clpY*), and adaptor proteins (*clpS*; green color). The intergenic regions are depicted as interrupted lines. The Clp AAA+ proteins have been grouped as class-I (ClpX, ClpY; orange color) and as the class-II chaperone (ClpA, ClpB, ClpC; grey color) based on the number of ATPase domain(s).

Table 2. 1 Caseinolytic protease (*clp*) genes location in *Leptospira* genome

Gene (Locus tag)	Gene coordinates (NCBI accession no. NC_005823.1)	UniProt accession no.	Gene size (bp)	Intergenic region (bp) (between successive *clp* genes)
clpC (*LIC10339*)	384088 – 386628	Q72VF8	2541	1283538 (*clpC* & *clpS*)
clpS (*LIC11356*)	1670167 – 1670487	Q72SM3	321	72860 (*clpS* & *clpP1*)
clpP1 (*LIC11417*)	1743348 – 1743944	Q72SG6	597	10 (*clpP1* & *clpX*)
clpX (*LIC11418*)	1743955 – 1745217	Q72SG5	1263	214309 (*clpX* & *clpQ*)
clpQ (*LIC11600*)	1959527 – 1960069	Q72RY8	543	9 (*clpQ* & *clpY*)
clpY (*LIC11601*)	1960079 – 1961518	Q72RY7	1440	245082 (*clpY* & *clpA*)
clpA (*LIC11814*)	2206601 – 2208841	Q72RD2	2241	4 bp overlap (*clpA* & *clpS*)
clpS (*LIC11815*)	2208838 – 2209173	Q72RD1	336	150158 (*clpS* & *clpP2*)
clpP2 (*LIC11951*)	2359332 – 2359925	Q72R01	594	68240 (*clpP2* & *clpB*)
clpB (*LIC12017*)	2428166 – 2430748	Q72QU2	2583	Not applicable

It has been previously reported that the genes encoding caseinolytic proteases are highly conserved in both saprophytic and pathogenic strains of *Leptospira* and comprise a part of the core group of genes in the genus *Leptospira* (Picardeau et al. 2008). The two core catalytic components ClpP1 and ClpP2, are the paralogs of peptidase ClpP, a serine protease, which is predicted to perform the proteolysis activity mainly in association with the regulatory proteins. ClpA, ClpB, ClpC, ClpX, and ClpY are the chaperone proteins belonging to the Clp/Heat shock protein 100 (Hsp100) family that carry AAA+ domains, a characteristic of the ATPases superfamily. These Clp ATPase subunits have been clustered as the class-I and class-II chaperone based on the number of AAA+ domain it carries. The class-I chaperone (ClpX and ClpY) in *Leptospira* carries one AAA+ domain, whereas the class-II chaperone (ClpA, ClpB, and ClpC) carries two AAA+ domains (**Figure 2.1**). Among the class-II chaperone, ClpB lacks a binding motif (P-loop motif) at the C-terminal essential for forming Clp proteolytic complex. Therefore, the *Leptospira* ClpB mainly mediates protein disaggregation with or without the DnaK chaperone system's co-operation and is established to be a virulence factor in pathogenic

Leptospira (Krajewska et al. 2017, Lourdault et al. 2011). The deletion of the *clpB* chaperone in *Leptospira* exhibited impaired growth under stress conditions (Lourdault et al. 2011). In the *Leptospira* genome, the genes *clpP1* and *clpX* are located close to each other with an intergenic gap of 10 base pairs (bps), whereas *clpP1* and *clpP2* are separated by 34 different genes (**Table 2.1**). Based on the location of the genes encoding ClpP isoforms in the *Leptospira* genome, these *clpP* genes may be independently transcribed and regulated. Using quantitative real-time polymerase chain reaction (qRT-PCR), the analysis of mRNA transcripts of *clpP* isoforms in *Leptospira* demonstrated each gene to be differentially transcribed (**Appendix A.1**). In *Leptospira* spp., the existence of two paralogs of the *clpS* gene was also predicted, where one (*LIC11815*) of the *clpS* genes lies adjacent to *clpA* (*LIC11814*), whereas the other (*LIC11356*) *clpS* is located distant apart on the chromosome (**Figure 2.1** and **Table 2.1**). The catalytic unit ClpQ, a threonine protease of *Leptospira,* has been established previously to interact with the chaperone ClpY to form the functional ClpYQ protease (also known as HslUV) (Dong et al. 2017). Moreover, in a previous study, it is proposed that regulatory proteins ClpX, ClpA, and ClpC of *Leptospira* have a binding motif (P-loop motif) at the C-terminal that may interact with the N-terminal loop of ClpP and form the Clp proteolytic complex . Therefore, it was interesting to study how multi-isoforms of ClpP in *Leptospira* interact with its chaperone to form the operative proteases.

2.3.2 Molecular characterization of core catalytic component ClpP

The open reading frames (ORFs) of full-length *clpP1* (597 bp), *clpP2* (594 bp), and *clpX* (1263 bp) were amplified by PCR using genomic DNA as a template of *L. interrogans*. The PCR amplicons of *clpP1*, *clpP2,* and *clpX* were cloned separately in the pET23a expression vector, and the recombinant proteins were overexpressed in *E. coli* BL21 (DE3) cells (**Figure 2.2A**). The overexpressed recombinant proteins (LepClpP1, 23 kDa; LepClpP2, 22 kDa; and LepClpX, 47 kDa) of *Leptospira* were purified using Ni-NTA affinity column chromatography (**Figure 2.2 B**).

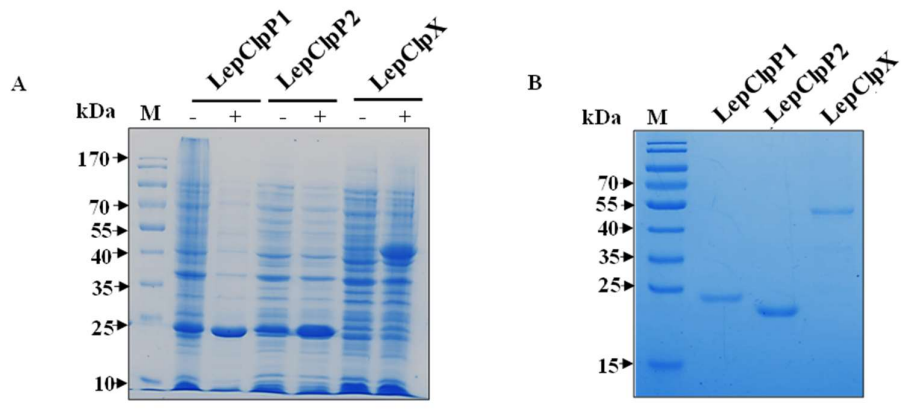


Figure 2. 2 Purification of leptospiral recombinant LepClp (LepClp) proteins.
(A) Cloning and overexpression of LepClpPs and LepClpX in *E. coli*. The Coomassie-blue stained 12% SDS-polyacrylamide gel showing the resolved protein lysates of *E. coli* cells overexpressing *Leptospira clpP1* (pET23a-clpP1), *clpP2* (pET23a-clpP2) and *clpX* (pET23a-clpX), after induction with (+) or without (-) 1 mM IPTG. (B) The affinity purified Leptospira recombinant proteins. The Ni-NTA affinity purified LepClpP1 (~ 23 kDa), LepClpP2 (~ 22 kDa) and LepClpX (~47 kDa) proteins were resolved on 12% SDS-polyacrylamide gel and stained with Coomassie-blue. In image A and B, M denotes the standard protein molecular size marker (in kDa).

The purified LepClpP1 and LepClpP2 were used to generate polyclonal antibodies in rabbit and BALB/c mice, respectively. *In silico* analysis of the available amino acid (aa) sequence of *clpP1* and *clpP2* in *L. interrogans* Copenhageni exhibits 100% identity to its orthologs in another pathogenic serovar, *L. interrogans* Lai (*clpP1-LA2559; clpP2-LA1953*), whereas non-pathogenic serovar *L. biflexa* Patoc, possesses only 44.1% *(clpP1-LEPBI_I1760*) and 42.5% (*clpP2-LEPBI_I0969*) identities. The ClpP isoforms in the *Leptospira* (serovars Lai and Copenhageni) lysates were detected at the comparable molecular size to that of recombinant protein on immunoblotting, and no remarkable detection was observed in the serovar Patoc (**Figure 2.3 A** and **2.3 B**). Immunoblots' result of the LepClpP isoforms in *Leptospira* serovars is in agreement with the *in silico* findings. We also validated the cross-reactivity of the generated polyclonal antibodies towards each LepClpP isoforms of *Leptospira* as they show 42% identities within themselves. To our surprise, a cross-reactivity of anti-LepClpP1 antibodies with LepClpP2 using immunoblots was detected; however, anti-LepClpP2 antibodies were specific (**Figure 2.3 C** and **2.3 D**).

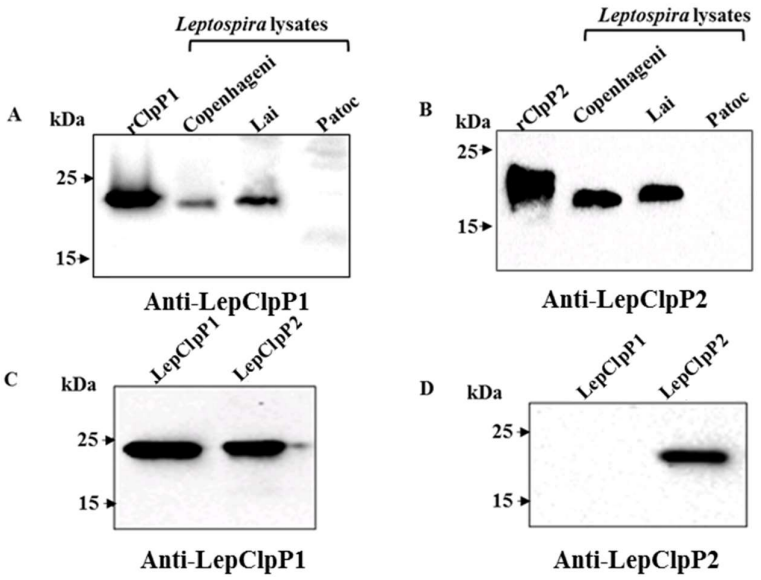


Figure 2. 3 Immunoblotting of native ClpP component of *Leptospira* in pathogenic and non-pathogenic serovars.
(**A and B**) Western blot of native ClpP1 and ClpP2 of *Leptospira,* respectively. The native ClpP1 and ClpP2 in *Leptospira* lysates were detected in pathogenic strains (serovar Lai and Copenhageni) but not in the non-pathogenic strain (serovar Patoc) using the primary antibodies generated against LepClpP1 and LepClpP2, respectively. Native and recombinant LepClpP isoforms were detected at the similar molecular size. (**C**) Cross-reactivity of anti-LepClpP1 with LepClpP2. Western blot of LepClpP1 and LepClpP2 with anti-LepClpP1 revealed that anti-LepClpP1 could cross-react and detect LepClpP2. (**D**) Specific detection of LepClpP2 with anti-LepClpP2. Western blot of LepClpP1 and LepClpP2 with anti-LepClpP2 showed that anti-LepClpP2 was specific unlike anti-LepClpP1.

2.3.3 *Leptospira* ClpP1 and ClpP2 coupling are essential for cleaving small peptide substrates

The multi-ClpP isoforms tend to reveal structural and functional disparity in an organism-specific manner (Pan et al. 2019). Only 42% percent identity exists within the *Leptospira* ClpP isoforms. Such observation incites query whether single or both ClpP isoform(s) would form active tetradecameric complexes capable of peptide/protein degradation. The catalytic triad Ser-His-Asp alignment of ClpP in extended conformation is capable of degrading small peptides (<5−6 amino acids) without the requirement of its chaperone or ATP (Woo et al. 1989). Therefore, the capability of pure LepClpP isoforms to hydrolyze fluorogenic di- and tetra-peptides such as Suc-LY-AMC (Woo et al. 1989) (S1) and Suc-LLVY-AMC (Woo et al. 1989) (S2), respectively, and a Fluorescein isothiocyanate-labeled full-length casein protein substrate (Li et al. 2016) (FITC-casein, S3) were evaluated. Unexpectedly, the pure LepClpP isoforms of *Leptospira* was enzymatically inactive on the fluorogenic small peptide substrates (S1 and S2) till 1 h of the reaction (**Figure 2.4 A**). For further validation, the peptidase reaction

time was extended to 48 h, but no peptidolytic activity was measured using the pure LepClpP isoforms (data not shown). The possibility of N-terminal pre-processing of ClpP isoforms of *Leptospira* to produce mature ClpP peptidase was not determined as there are other reported pathogenic bacterial species like *Listeria* (Zeiler et al. 2013), *Clostridium* (Lavey et al. 2018), *Pseudomona*s (Hall et al. 2016) and *Chlamydia* (Wood et al. 2018) whose pure ClpP isoforms showed functional activity without any pre-processing. However, when pure LepClpP isoforms of *Leptospira* were incubated together in equimolar concentration for 10 min at 37°C, the resulting LepClpP isoforms mixture could hydrolyze the fluorogenic small peptide substrates (S1 and S2) at variable rates (**Figure 2.4 A**). The substrate S1 was selected to determine the enzyme kinetic parameters of the LepClpP isoform mixture due to its faster rate of hydrolysis and accepted model peptide substrate for the ClpP protease study. Unlike other reported enzyme kinetics of the functional ClpP (Dong et al. 2017, Lavey et al. 2018), which show Michaelis-Menten kinetics, LepClpP isoforms mix exhibit Hill kinetics on fluorogenic substrate S1. The substrate S1 cleavage saturation curve by LepClpP isoforms mixture was sigmoidal with a V_{max} of 2531 ± 114 ΔRFU/min and apparent K_M (K_M") of 0.87 ± 0.05 mM. The dependency on the substrate (S1) concentration for increased peptidase activity of LepClpP isoforms reflected a slightly cooperative mechanism with a Hill coefficient (H) of 3.53 ± 0.62 (**Figure 2.4 B**). This suggests that the multiple molecules of the substrate (S1) bind to the LepClpP1P2 mixture of *Leptospira* and stimulate its activity, in addition to being its substrate. Previously, activation of such an inactive ClpP1P2 complex of *M. tuberculosis* by peptide activators (Z-Leu-Leu and Z-Leu-leucinal) in the hydrolysis of Z-Gly-Gly-Leu-AMC (0.1 mM) and Ac-Nle-Pro-Nle-Asp-AMC (0.1 mM) substrates followed a highly cooperative mechanism (H= 5 -7) (Akopian et al. 2012). Additionally, in agreement with the earlier evidence of ClpP biochemical function in other pathogens (Kress et al. 2009), no protease activity was recorded on the FITC-casein substrate (S3) by the LepClpP isoforms mixture of *Leptospira* (data not shown). These multiple ClpP isoforms tend to exhibit the functional disparity in an organism-specific manner (Gaillot et al. 2000, Hall et al. 2016, Ravikiran M. Raju et al. 2012, Wood et al. 2018). In a recent study on *Clostridium difficile,* both ClpP isoforms are capable of forming functional peptidase independently (Lavey et al. 2018). Our LepClpP isoform peptidase activity results agree to *M. tuberculosis,* where too both isoforms are inactive towards the dipeptide substrate (Akopian et al. 2012). It is also possible that pure LepClpP isoforms may show activity independently on other types of small peptides that are yet to be evaluated. In *L. interrogans,* coupling of each LepClpP isoforms ensures to achieve peptidase activity on small model peptides substrates (S1 and S2), whereas activity on the large

protein substrate (S3) may require additional unfolding by its co-chaperone. Although the gene encoding each ClpP isoforms of *Leptospira* are chromosomally far apart and show lower sequence identity, this assay provided us a clue that pure LepClpP1 and LepClpP2 may interact and self-assemble into functional heteromeric peptidase complex.

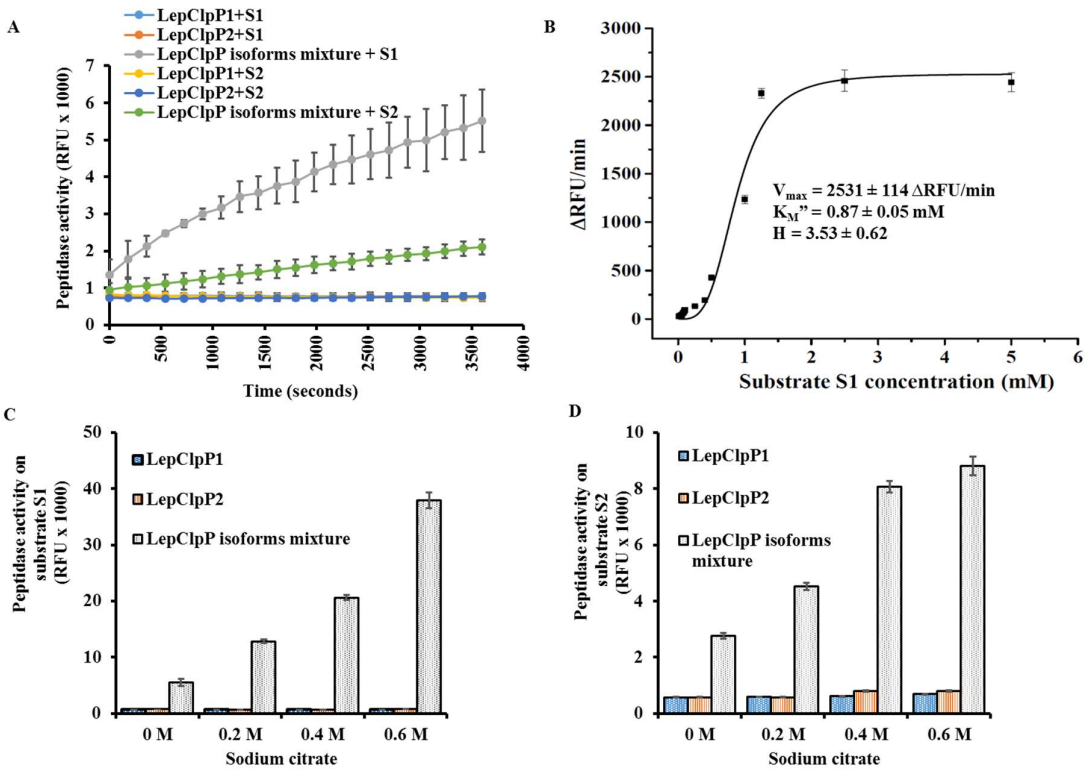


Figure 2.4 Peptide degradation assays using recombinant ClpP (LepClpP) isoforms of *Leptospira*.
(A) Peptidase activity of LepClpP isoforms on the small fluorogenic peptide substrates S1 and S2. Peptide degradation was measured fluorometrically as Relative Fluorescent Unit (RFU X 1000) using the fluorogenic substrates, Suc-LY-AMC (S1) and Suc-LLVY-AMC (S2) for 1 h. The pure LepClpP isoforms are inactive on small peptide substrates (S1 and S2) whereas the LepClpP isoforms mixture is enzymatically active. **(B)** Peptidase kinetics of LepClpP isoforms on fluorogenic substrate S1 determined using Hill function. The apparent K_M" or the substrate concentration corresponding to the half-maximal velocity of the reaction (K_M"= 0.87 ± 0.05 mM), maximal reaction velocity (V_{max}= 2531 ± 114 ΔRFU/min), and the Hill coefficient (H= 3.53 ± 0.62) of LepClpP isoforms mixture during fluorogenic substrate S1 hydrolysis was determined by the non-liner curve fitting function of the Origin Software. **(C and D)** Effect of sodium citrate on the peptidase activity of LepClpP isoforms mixture on fluorogenic substrate S1 and S2, respectively. Gradient increase in the sodium citrate concentration stimulates the peptidase activity of LepClpP isoforms mixture but not of the pure LepClpP isoforms. The error bars represent the standard deviations (SDs) from the two independent experiments performed in duplicates.

We next assessed whether the activity of LepClpP isoforms mixture could be enhanced in the presence of sodium citrate, a salt that promotes oligomeric complex stabilization and has been reported to increase ClpP peptidase activity (Gersch et al. 2016, Hall et al. 2016). It is noted

that salts like sodium citrate have high Hofmeister strength causing a "salting out" effect and stabilizing functional heteromeric complexes. Interestingly, with the gradient increase in sodium citrate concentration (0.2 - 0.6 M), there was an increase in peptidase activity of LepClpP isoforms mixture on the model peptide substrates (S1 and S2), whereas the pure LepClpP1 and LepClpP2 did not show any gain in peptidase activity (**Figure 2.4 C** and **2.4D**). The effect of sodium citrate on LepClpP peptidase activity was in agreement with other reported ClpP multi-isoforms (Gersch et al. 2016, Wood et al. 2018).

2.3.4 *Leptospira* ClpP1 and ClpP2 display optimum peptidase activity at 1:1 stoichiometry

As we showed, each LepClpP isoform in combination is essential for peptidase activity on smaller peptide substrates (S1 and S2). It captured our interest in identifying the optimum molar-ratio of pure ClpP isoforms required for heterocomplex formation. Isothermal Titration Calorimetry (ITC) is a well-established method employed to study protein-protein interactions and estimate the stoichiometry of the heterocomplex (Pierce et al. 1999, Velazquez-Campoy et al. 2015, Ka et al. 2016, Nuñez et al. 2014). Therefore, the molar ratio required for forming heterocomplex between the pure LepClpP isoforms of *Leptospira* was determined using ITC measurements. The ITC data demonstrate that one molecule (n = 0.945) of LepClpP1 was bound to 1 molecule of pure LepClpP2 with a dissociation constant (K_d) of 2.02 ± 0.1 µM at 37ºC and is thermodynamically favored (**Figure 2.5 A**). Moreover, the measured K_d value demonstrates a moderate binding affinity between the two LepClpP isoforms, and therefore, the coupling of LepClpP isoforms, and its stabilization may be time-dependent. In support of ITC data, an additional peptidase assay was performed with varying ratios of pure LepClpP isoforms mixture required to exhibit the maximal activity. Upon increasing the amounts of pure LepClpP1 (0-2.5 µg) with a constant amount of pure LepClpP2 (1.0 µg) in the mixture, the peptidase activity gradually increased on substrate S2. The peptidase activity on fluorogenic substrate S2 (tetrapeptide) reached its optimum when the equimolar ratio (1.1 -1.2) of LepClpP1 and LepClpP2 was mixed (**Figure 2.5 B**). Such finding was in agreement with the reported optimum peptidase activity of multi-isoforms of ClpP in *Mycobacterium* (Akopian et al. 2012). These results suggest that the maximum peptidase activity on substrate S2 is achieved when a mixture of pure lepClpP1 and LepClpP2 isoforms acquires 1:1 stoichiometry under the given *in vitro* condition.

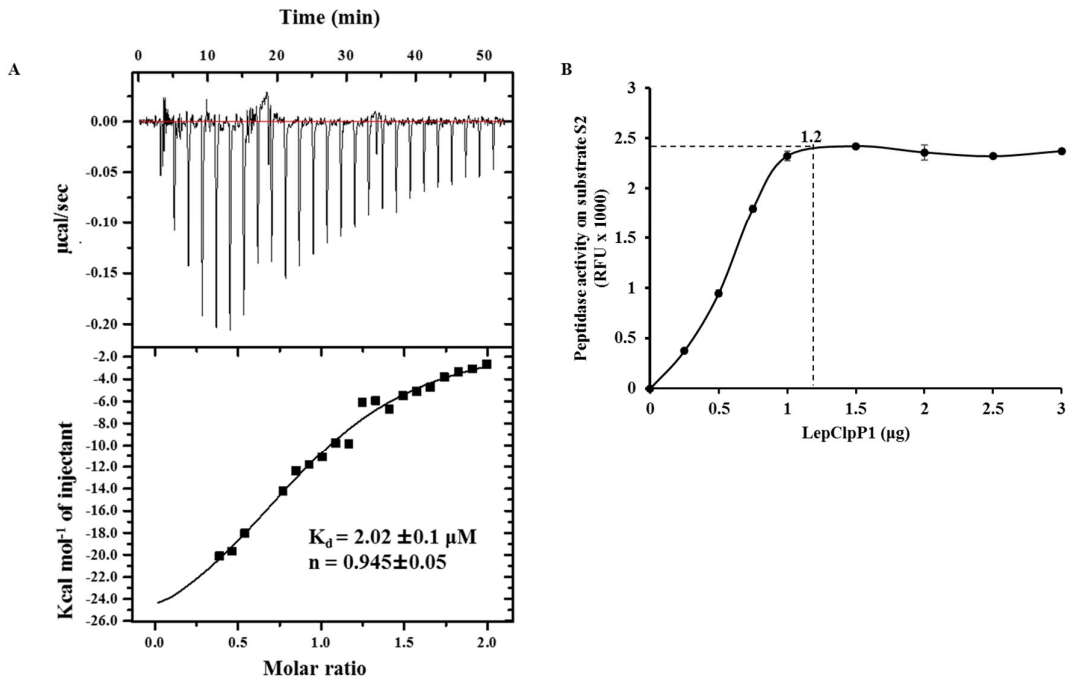


Figure 2. 5 The *Leptospira* ClpP isoforms mixture interacts in equimolar ratio for optimal peptidase activity.

(A) Isothermal Titration Calorimetry (ITC) of pure LepClpP1 and LepClpP2. One molecule of LepClpP1binds with one molecule of LepClpP2 at 37ºC. Parameters of the ITC experiment obtained includes stoichiometry factor (n) = 0.945 ± 0.05; dissociation constant (K_d) = 2.02 ± 0.1 µM; enthalpy (ΔH) = -30220 ± 2636 cal^{-1} mol^{-1}; entropy (ΔS) = -71.4 cal^{-1} mol^{-1} K^{-1}. **(B)** Optimum peptidase activity of LepClpP mixture on fluorogenic substrate Suc-LLVY-AMC (S2). Constant amounts of pure LepClpP2 (1 µg) were mixed with increasing amounts of pure LepClpP1 and hydrolysis of fluorogenic substrate S2 was measured as Relative Fluorescent Unit (RFU X 1000). The maximal activity of the LepClpP isoforms mixture is indicated by the dotted lines in the graph. The error bars represent the standard deviations (SDs) from the two independent experiments performed in duplicates.

2.3.5 The LepClpP1 and LepClpP2 subunits re-arrangement for composing functional LepClpP protease

The size-exclusion chromatography (SEC) of the pure LepClpP isoforms or its mixture was performed to determine if these tend to oligomerize as reported in other ClpP of bacteria (Akopian et al. 2012, Hall et al. 2016). The SEC analysis demonstrated that LepClpP isoforms mixture tends to elute as tetradecamer precisely like the pure LepClpP2 (22 kDa × 14 subunits ~ 308 kDa), whereas the pure LepClpP1 displays a broad elution profile indicating higher oligomeric species in addition to the tetradecamer (**Figure 2.6 A**). The pure LepClpP isoforms also demonstrated a possible heptameric species (~200 kDa) as a small shoulder peak during SEC (**Figure 2.6 A**). Such oligomerization property was also observed when the pure LepClpP isoforms or its mixture were resolved on native-PAGE and stained with Coomassie Blue (**Figure 2.6 B**). However, the oligomeric forms of LepClpP1 and the heterocomplex mixture

on native-PAGE showed a discrepancy in size with respect to the SEC elution profile. The *Leptospira* pure LepClpP1 on native-PAGE got resolved in a majority at a higher order of oligomeric species (~ 480 kDa; 21-mer) and the pure LepClpP2 resolved at the tetradecameric form (~ 308 kDa). In SEC and native-PAGE gel, the proteins migrate according to their size and shape and hydrodynamic properties. Such a difference in migration behavior of protein might explain the inconsistency between the sizes of the LepClpP oligomers determined. To further validate, the pure LepClpP isoforms and its mixture were incubated for a short period (1 h) and an extended period (48 h) at 4°C to allow time for subunits re-arrangement, and after that, resolved on the Native-PAGE. A shift in the mobility of LepClpP1 to an available tetradecamer size from 21-mer species was detected on Native-PAGE in the LepClpP isoforms mixture after 48 h of incubation, but not in the short period incubated LepClpP isoforms mixture (**Appendix A.2**). In contrast, the pure LepClpP1 resolved at 21-mer species even after 48 h of incubation, an observation that is in agreement with the finding under SEC.

It is to be noted that the pure LepClpP1 and LepClpP2 are functionally inactive and are fractionating by SEC empirically at ≥ 308 kDa (14-21-mer) and 308 kDa (tetradecamer), respectively, whereas the functionally active LepClpP isoforms mixture fractionates as tetradecamer (**Figure 2.6 A** and **Appendix A.2**). The functional activity in LepClpP protease is attained only when both the isoforms contribute together to frame the tetradecameric structure where the catalytic triad is aligned (i.e., active) in an extended conformation. Moreover, since the ITC result demonstrates that LepClpP1 and LepClpP2 bind in a 1:1 stoichiometry, the active LepClpP protease (14-mer) macromolecule may be composed of 2-stacked heptameric rings (7+7-mer) formed by homogenous subunits of LepClpP1 and LepClpP2. It is also conceivable that each heptameric ring contains a mixture of LepClpP1 and LepClpP2 subunits, as has been reported in the *Synechococcus* ClpP complexes (Stanne et al. 2007, Andersson et al. 2009). The subunits of pure LepClpP isoforms or its mixture were chemically cross-linked using glutaraldehyde to determine the LepClpP heptameric ring's composition. After 15 min of cross-linking reaction, distinct bands of cross-linked LepClpP1 intermediates corresponding to 1-, 2-, 4-, 5-, 6- and 7-mers on molecular weight were evident on SDS-PAGE stained with Coomassie Blue (**Figure 2.6 C**). Similar patterns of oligomeric bands of LepClpP2 corresponding to 1-, 2-, 5-, 6- and 7-mers were also evident after 15 min of chemical cross-linking reaction (**Figure 2.6 D**). With the increasing time of chemical cross-linking, the intensity of transient forms of pure LepClpP1 and LepClpP2 cross-linked bands were observed with reduced intensity of protomers after gel electrophoresis. Moreover, when the LepClpP isoforms mixture was chemically cross-linked, bands of cross-linked products

identical to pure LepClpP isoforms were observed, suggesting each heptameric form of pure LepClpP isoforms constitute the active tetradecameric structure of the LepClpP protease (**Figure 2.6 E**).

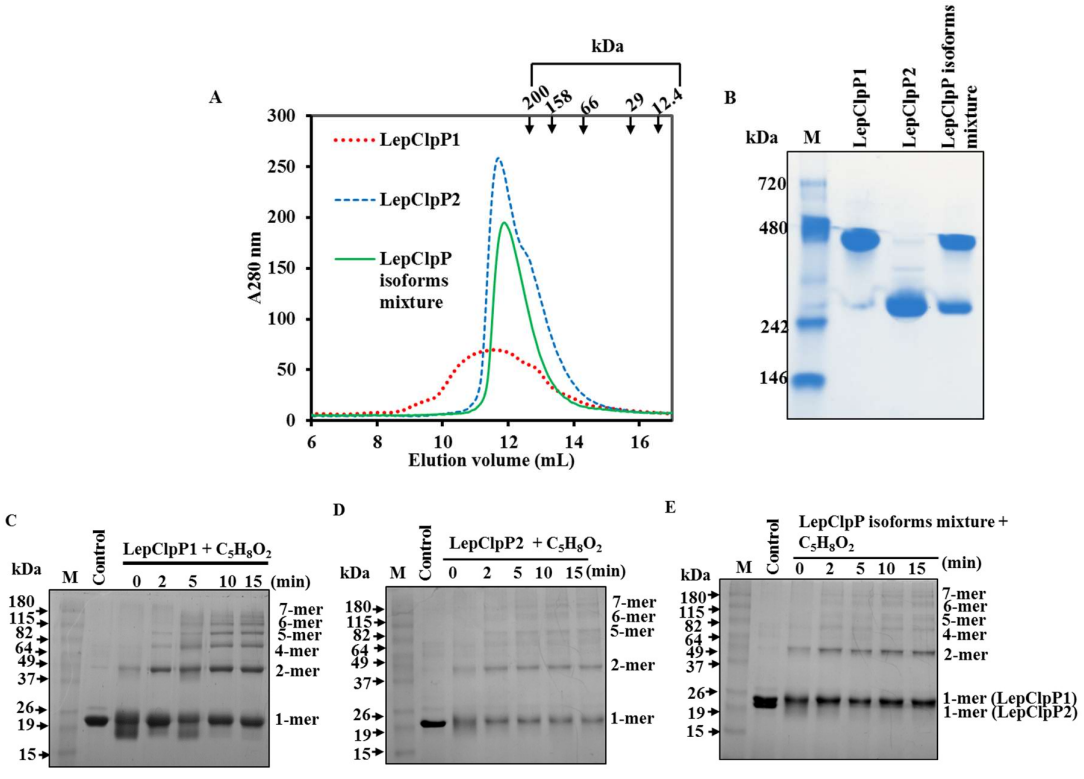


Figure 2. 6 Oligomerization of LepClpP isoforms.
(A) Size exclusion chromatography of pure LepClpP isoforms and its mixture. The oligomeric states of pure LepClpP isoforms and its mixture was monitored by size exclusion chromatography using an Enrich 650 High-Resolution column. Elution positions of the standard proteins with its molecular weight are indicated in arrows pointing downward. The molecular weight standard protein markers in kiloDalton (kDa) includes β-amylase (200), alcohol dehydrogenase (158), albumin (66), carbonic anhydrase (29) and cytochrome C (12.4). The pure LepClpP2 and the LepClpP isoforms mixture were eluted as tetradecamer (308 kDa) whereas the pure LepClpP1 display a broad elution profile (≥ 308 kDa). A small shoulder peak corresponding to 200 kDa was also seen for pure LepClpP1 and LepClpP2. **(B)** Pure LepClpP isoforms or its mixture resolved on the native-polyacrylamide gel. The purified LepClpP isoforms or its mixture (2 µg) were resolved on a 4-20% native-PAGE and stained with Coomassie-blue. The standard molecular weight marker was run in lane 1 (M). The purified LepClpP1 and LepClpP2 resolved at around 480 kDa (23 kDa × 21= 483 kDa) and 308 kDa (22 kDa×14= 308 kDa), respectively whereas the LepClpP isoforms mixture resolved at 480 and 308 kDa. **(C)** Denaturing polyacrylamide gel electrophoresis of pure LepClpP1 after glutaraldehyde ($C_5H_8O_2$) cross-linking. Chemical cross-linking of pure LepClpP1 with glutaraldehyde show distinct higher molecular weight bands that gets intensified with time (0-15 min) **(D)** Denaturing polyacrylamide gel electrophoresis of pure LepClpP2 oligomeric subunits after glutaraldehyde cross-linking. Chemical cross-linking of pure LepClpP2 with glutaraldehyde ($C_5H_8O_2$) show distinct higher molecular weight bands in comparison to control (without $C_5H_8O_2$). **(E)** Denaturing gel electrophoresis of the LepClpP isoforms mixture after glutaraldehyde cross-linking. The cross-linked subunits pattern of LepClpP isoforms mixture is similar to that of pure

LepClpP isoforms. The denaturing polyacrylamide gels were stained with Coomassie-blue and for clarity, the image obtained is represented after inversion.

Dynamic light scattering (DLS) was performed on pure LepClpP and its isoforms mixture to address the size distribution (hydrodynamic diameter) of the oligomeric species of LepClpP in solution. The pure LepClpP1 incubated either for 1 h or 24 h demonstrated two major peaks with similar hydrodynamic diameter by DLS. The representative two major peaks for pure LepClpP1 (24 h incubation) correspond to mean hydrodynamic diameters of 19.06±5.76 nm and 197.8±73.2 nm (**Figure 2.7 A**). On the contrary, only one population of oligomeric species of lower particle size (16.57±6.03 nm) was detected in pure LepClpP2 incubated either for 1 h or 24 h (**Figure 2.7 B**).

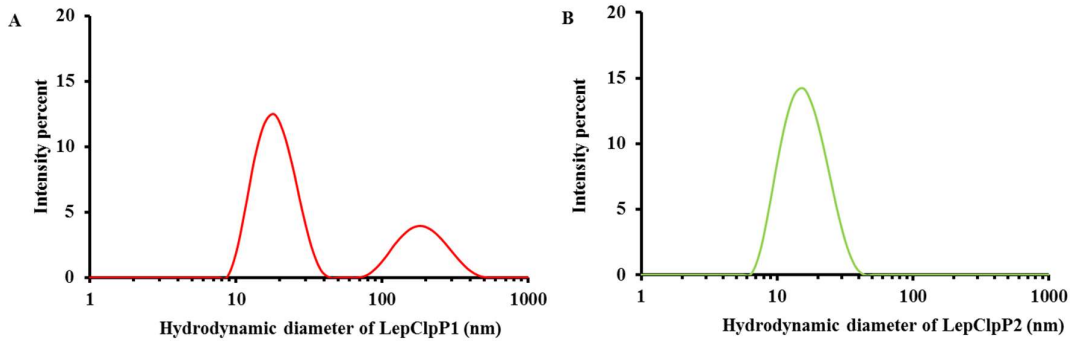


Figure 2. 7 Size distribution in LepClpP isoforms by dynamic light scattering (DLS). **(A)** The size distribution profile of oligomeric subunits in pure LepClpP1. The pure LepClpP1 after 24 h of incubation shows two major peaks of oligomeric species with the particle diameter of 19.06 ± 5.76 nm and 197.8 ± 73.2 nm. **(B)** The size distribution profile of oligomeric subunits in pure LepClpP2. The representative size distribution data in pure LepClpP2 after 24 h of incubation showed single oligomeric species with an average hydrodynamic diameter of 16.57 ± 6.03 nm. Consistent size distribution profile were obtained for pure LepClpP isoforms during short incubation (1 h) too.

The LepClpP isoforms mixture after a short incubation of 1 h for self-assembly showed two major peaks with mean hydrodynamic diameters of 19.06±5.29 nm and 312.5±113.7 nm (**Figure 2.7 C**). The DLS results demonstrated multiple populations of self-assembled oligomeric species for both pure LepClpP1 and the LepClpP isoforms mixture. Interestingly, LepClpP isoforms mixture after prolonged incubation (24 h) showed a shift in the oligomeric species from multiple to single populations with an average hydrodynamic diameter of 18.21±5.54 nm (**Figure 2.7 C**). For further substantiation, the obtained values of Polydispersity, Polydispersity index, and correlation function during DLS analysis of the pure LepClpP isoforms or its mixture (1 h or 24 h incubation) have been shown (**Appendices A.3** and **A.4**). Such a shift from multi-population of oligomeric species to a single population was accompanied by higher peptidase activity on fluorogenic substrate S1 than the LepClpP

isoforms mixture displaying multiple populations of oligomeric species formed during the short re-arrangement/self-assembly period (1 h) (**Figure 2.7 D**).

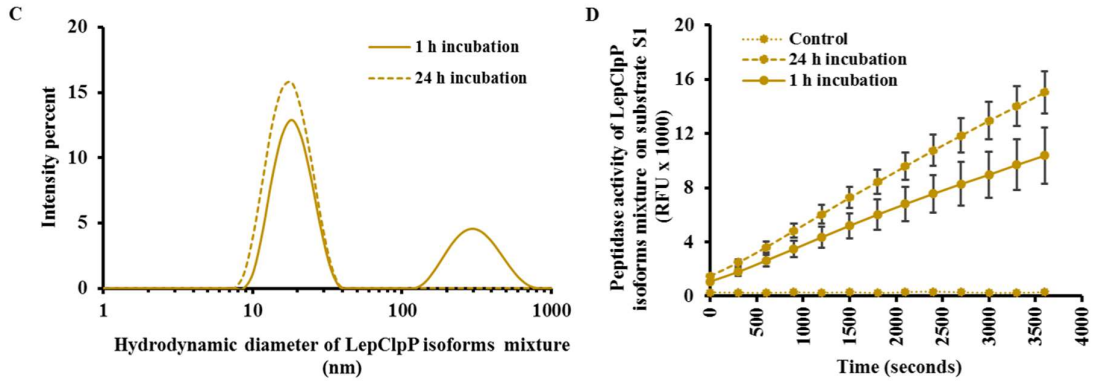


Figure 2.7. Size distribution in LepClpP isoforms by dynamic light scattering (DLS).
(C) The size distribution profile of oligomeric subunits in LepClpP isoforms mixture. The LepClpP isoforms mixture, when incubated for a longer duration (24 h) (dashed line), showed a shift in the size distribution of the oligomeric species to single population compared to the equivalent amount of LepClpP isoforms mixture incubated for a shorter duration (1 h) (solid line). The representative size distribution data of the 10 scans performed in duplicates are presented in Figure 7 A, B & C. **(D)** Effect on peptidase activity of the LepClpP isoforms mixture subsequent to a longer duration of LepClpP self-assembly. Incubation of LepClpP isoforms mixture for 24 h to self-assemble resulted higher (1.4 times) and statistically significant (p-value<0.05) peptidase activity on fluorogenic substrate S1 than the LepClpP isoforms mixture incubated for 1 h. Control denotes the background fluorescence of substrate S1 in the reaction.

The *Leptospira* ClpP isoforms being classified in the serine peptidase family, the effect of mutation of LepClpP isoforms (LepClpP1^{S98}A and LepClpP2^{S97}A) at one of the residues (Ser) of the catalytic triad was investigated. The complex of mutant LepClpP isoforms mixture (LepClpP1^{S98A}P2 and LepClpP1P2^{S97A}) did not demonstrate any peptidase activity on S1 and S2 substrates (**Figure 2.8 A**), even though mutant retained its ability to oligomerize like wild-type ClpP isoforms (**Appendix A.5**). In contrast, the complex of *L. monocytogenes* LisClpP2^{S98A} and LisClpP1 has been previously demonstrated a 75-fold increase in peptidase activity compared with pure LisClpP1 (Zeiler et al. 2013). The addition of sodium-citrate (0.6 M) to such complex of mutant LepClpP isoforms mixture failed to display any gain of peptidase activity on fluorogenic substrate S1 (**Figure 2.8 B**) and S2 substrates (data not shown). Thus, analysis of the pure LepClpP isoforms or its mixture macromolecule using various techniques suggests that the functional LepClpP protease in *Leptospira* is a tetradecamer and hypothesized to be composed of 2-heptameric rings of homogenous subunits of LepClpP1 and LepClpP2 where the proper alignment of all the 14-catalytic triad is essential.

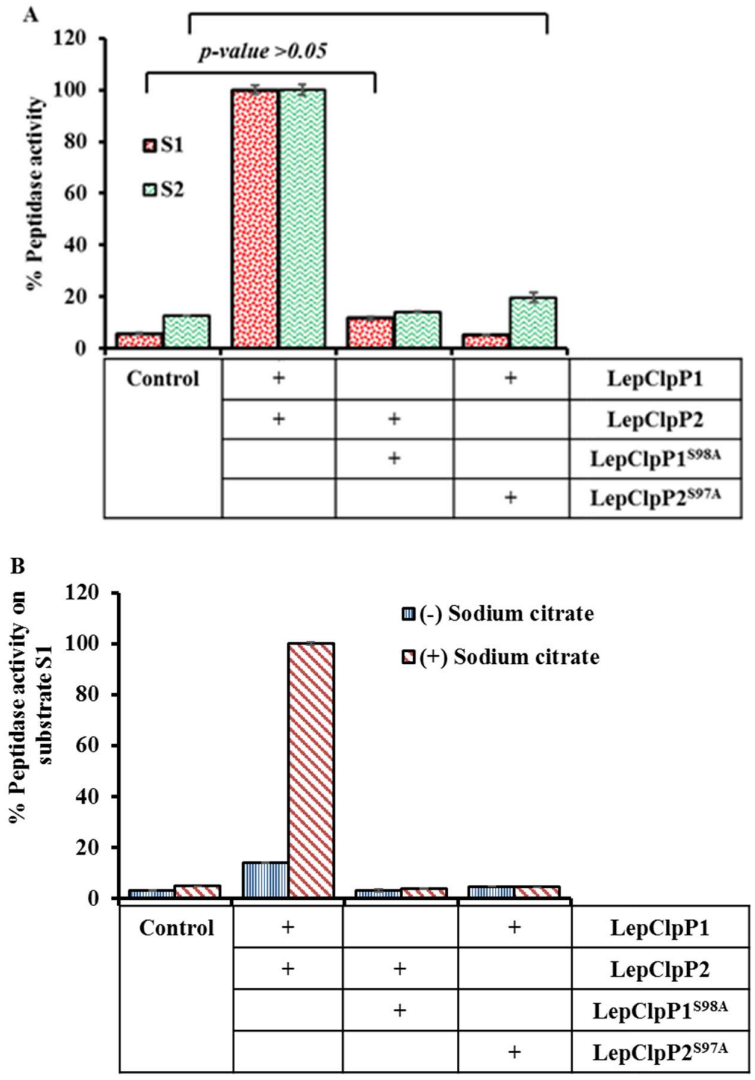
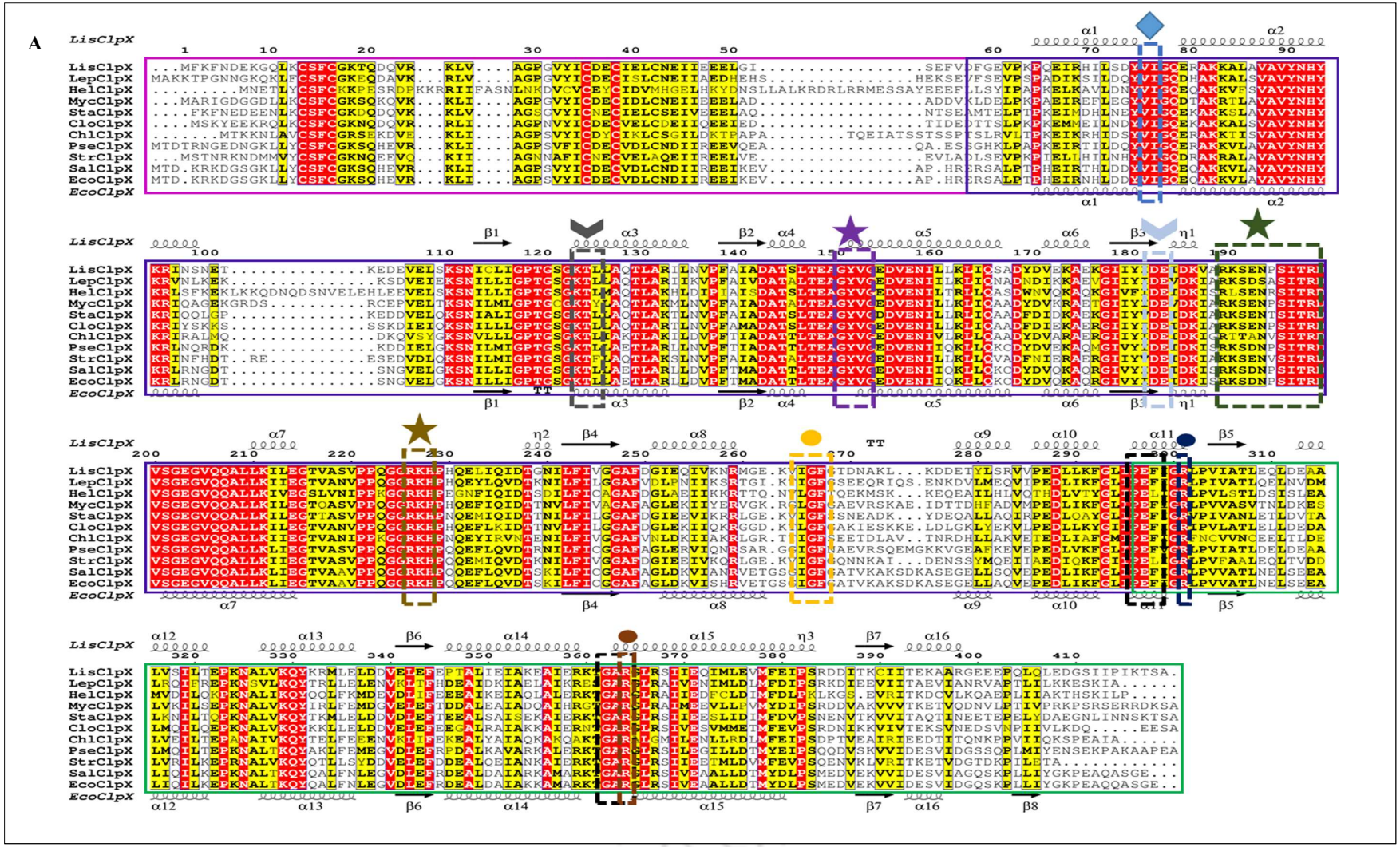


Figure 2. 8 Peptidase activity of the mutant LepClpP isoforms mixture.
Peptidase activity of LepClpP mixture is presented as a percentage (%), wherein, after 1 h of enzymatic reaction end-point fluorescence was measured. The measured end-point value of the wild-type LepClpP isoforms mixture was considered as 100% for measuring relative peptidase activity. Control shows the background fluorescence of the substrates in the reaction. **(A)** Comparison of peptidase activity of wild-type LepClpP isoforms mixture with mutant LepClpP isoforms mixtures on fluorogenic peptide substrates. Mutation of LepClpP isoforms ($LepClpP1^{S98A}$ and $LepClpP2^{S97A}$) was performed by substituting Ser98/97 to Ala98/97 residue in the catalytic triad. The LepClpP isoforms mixture generated in various combination with mutant and wild-type LepClpP isoforms showed complete loss of peptidase activity in mutant LepClpP isoforms mixture. **(B)** Effect of the presence of sodium citrate on peptidase activity of wild-type LepClpP isoforms mixture and mutant LepClpP isoforms mixtures. Presence or absence of sodium citrate (0.6 M) does not lead to gain in the peptidase activity in mutant LepClpP isoforms mixtures on fluorogenic peptide substrate S1. The error bars represent the standard deviations (SDs) from the two independent experiments performed in duplicates. Statistical analysis was performed by Student's t test for comparing the measured fluorescence value obtained for control and mutant LepClpP isoforms mixtures after 1 h of the of the assay (p-value>0.05).

2.3.6 The leptospiral ClpX retains conserved motifs of and exhibits predicted structural homology to, ClpX orthologs

We performed bioinformatics and *ab initio* structural modeling analyses to determine whether the leptospiral ClpX (LepClpX) possesses the expected conserved regions and motifs consistent with its proposed function as an AAA+ ATPase. Using Multiple Sequence Alignment (MSA), we aligned LepClpX to ClpX orthologs and elucidated the conserved motifs identified in other studies (**Figure 2.9 A**). LepClpX retains the N-terminal metal-binding domain (Wojtyra et al. 2003, Donaldson et al. 2003), the Box II, the Walker A and B motifs for ATP binding and hydrolysis (Walker et al. 1982, Wendler et al. 2012), the RKH motif and pore loops for substrate recognition (Siddiqui et al. 2004, A. Martin et al. 2008a, A. Martin et al. 2008b) and substrate unfolding (Iosefson et al. 2015b, Iosefson et al. 2015a), the arginine finger for inter-subunit sensing of nucleotide state in the ClpX hexamer (Wendler et al. 2012, Ogura et al. 2004), and the IGF loop for interaction with ClpP (Kim et al. 2001, Amor et al. 2019). We also constructed the domain structure of the LepClpX subunit based on the linear sequence of EcoClpX. The domain of ClpX subunits consists of a family-specific N-terminal domain, which is indispensable for degradation of ssrA-tagged proteins, and large and small AAA+ domains, which contain sequence motifs that mediate ATP binding and hydrolysis, ClpP binding, and substrate recognition (**Figure 2.9 B**) (Fei et al. 2019). The LepClpX is also predicted to form the expected homohexamer by structural modeling (**Figure 2.10 A**, two subunits removed for clarity). The spatial conservation of AAA and ClpX-specific motifs (colored in **Figure 2. 10 A** as in the MSA) indicates that the LepClpX likely functions using a similar or identical mechanism to that of other ClpX orthologs. Nevertheless, we acknowledge that without structural studies, it is too early to draw any conclusions about the conformational states of LepClpX.

The bioinformatics and homology modeling of LepClpX supports that it is a bonafide AAA+ ATPase. The MSA of LepClpX to other ClpX orthologs revealed high conservation of motifs involved in nucleotide binding, ATP hydrolysis, and nucleotide-state sensing (Martin et al. 2007, Singh et al. 2001). Taken together, these *in silico* studies suggest that LepClpX functions as a canonical AAA+ ATPase. The computational results thus further encouraged us to characterize the LepClpX biochemically.



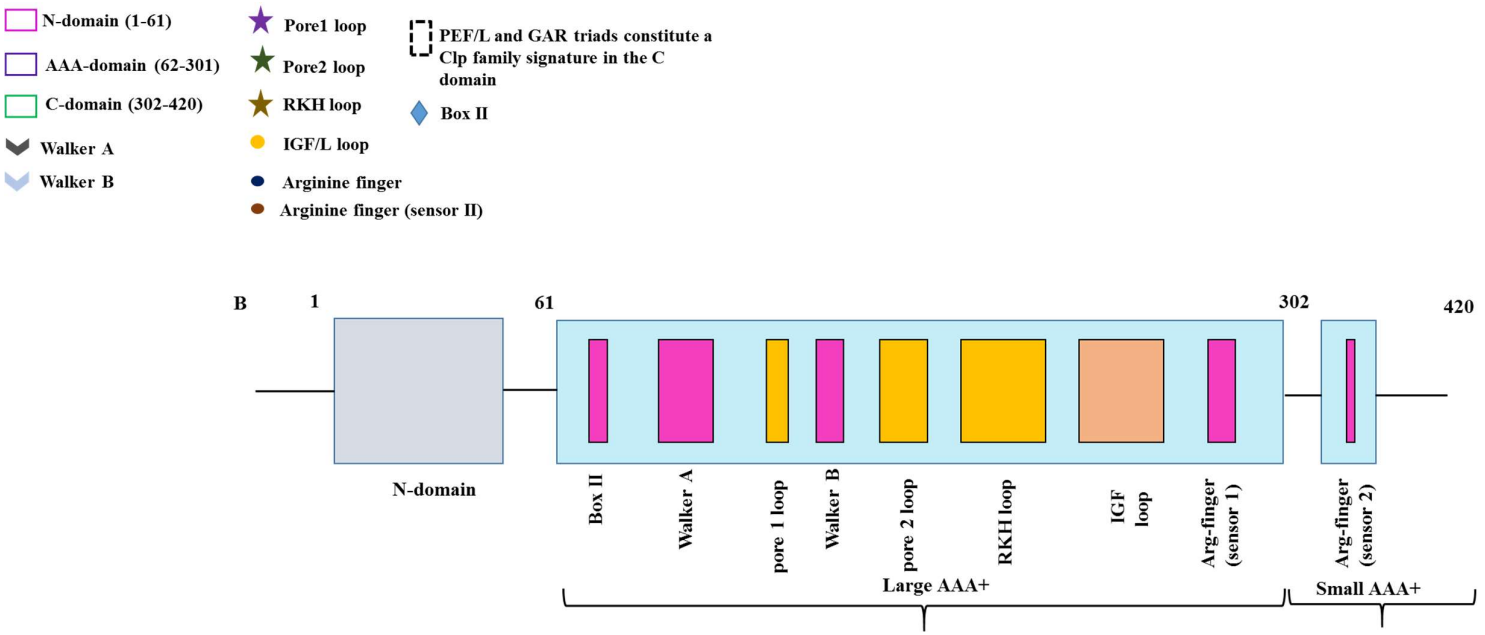


Figure 2. 9 Bioinformatic analysis of *Leptospiral* ClpX supports its role as an AAA+ ATPase.
(A) Multiple sequence alignment of LepClpX orthologs from various pathogenic bacteria was performed using Clustal Omega software. The ClpX orthologs used for amino acid alignment are, LisClpX: *Listeria monocytogenes* (Q8Y7K9), LepClpX: *Leptospira interrogans* Copenhageni (Q72SG5), HelClpX: *Helicobacter pylori* (O25926) MycClpX: *Mycobacterium tuberculosis* (P9WPB9), StaClpX: *Staphylococcus aureus* (Q2FXQ7), CloClpX: *Clostridium difficile* (Q180E8), ChlClpX: *Chlamydia trachomatis* (O84711), PseClpX: *Pseudomonas aeruginosa* (Q9I2U0), StrClpX: *Streptococcus pneumoniae* (P63792), , SalClpP: *Salmonella typhimurium* (Q87RC0), EcoClpX: *Escherichia coli* (P0A6Hi). The UniProt accession numbers are provided in parenthesis. Secondary structural elements present in the LisClpX (PDB ID: 6sfw) and EcoClpX (PDB ID: 3hte) structure are shown on the top and bottom of the sequence alignment, respectively. Identical and semi-identical residues in ClpX ATPases are highlighted in red and yellow colors, respectively, with residue numbers at the top of the alignment after LisClpX. The conserved key structural motifs in the ClpX subunits, including the Box II motif, Walker A and B motifs, RKH and pore loops, Arginine fingers, PEF/L and GAR triads in different colored boxes and marked with specific symbols at the bottom of the box. **(B)** LepClpX domain structure and positions of sequence motifs important for ATP binding and hydrolysis (magenta), substrate binding (yellow), and ClpP binding (orange), drawn on the basis of linear sequence of EcoClpX.

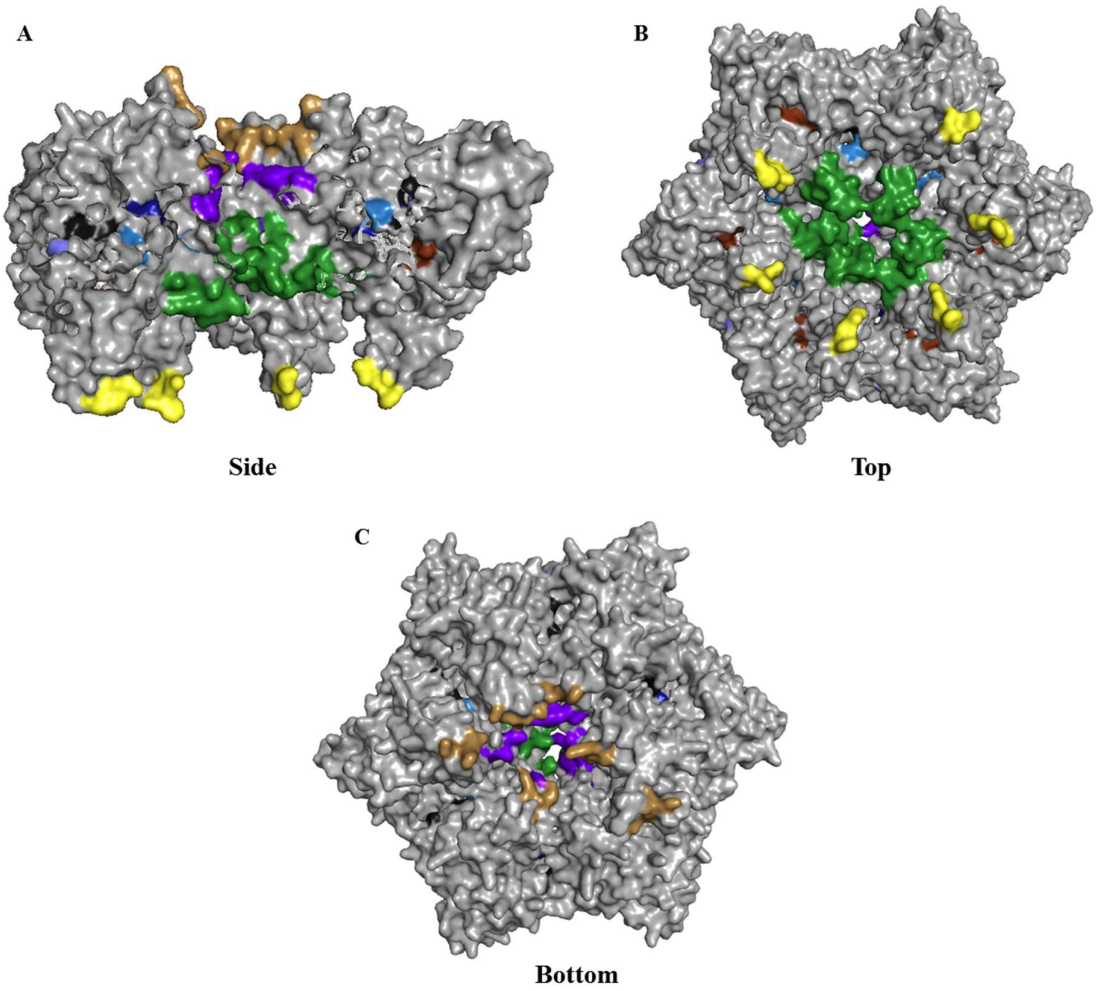


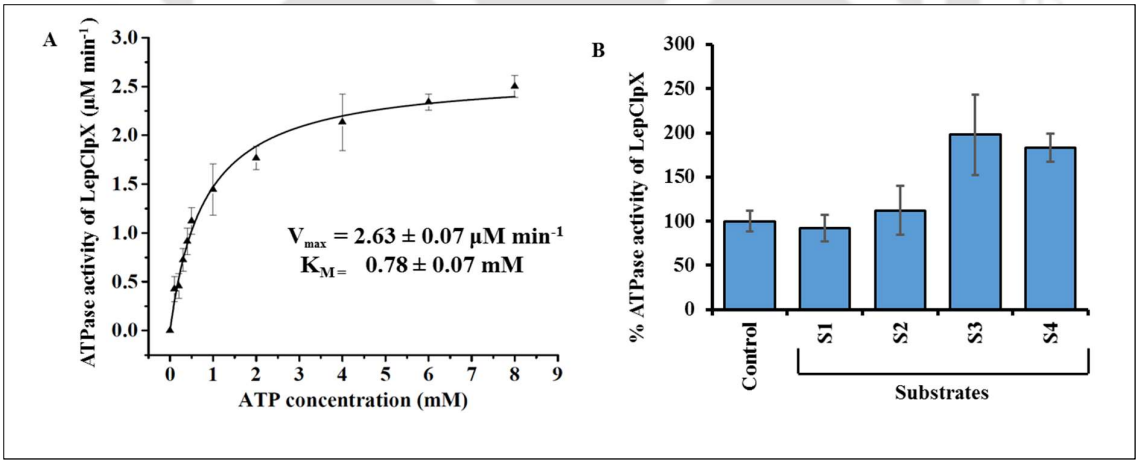
Figure 2. 10 Homology modeling of ClpX of *Leptospira* (LepClpX).
(**A, B, and C**) 3D model of LepClpX was generated using SWISS-MODEL and presented using the PyMol software. The side, top, and bottom surface representations of the model LepClpX hexamer, are depicted, respectively. The conserved key structural motifs in the LepClpX subunits, including the Box II motif, Walker A and B motifs, RKH and pore loops, Arginine fingers are colored maintaining the color scheme of panel A in **figure 2.9**. The LepClpX was modelled based on known crystal structure of *Listeria monocytogenes* ClpX (LisClpX, PDB id: 6sfw) as LepClpX has the highest sequence identity of 64% with LisClpX among all the compared ClpX orthologs in the MSA. The two subunits in the side view of the LepClpX were hidden for easier visualization of the conserved motifs into the complex.

2.3.7 The LepClpX stimulates LepClpP heterocomplex for protein degradation in an energy-dependent process

The overexpressed and purified LepClpX was assessed for its ATPase activity, an activity essentially required to unfold and permit the unfolded larger protein substrate to pass through the axial pores of ClpP protease (Kress et al. 2009). Moreover, ClpX oligomerization is stabilized by ATP; therefore, the assembly can be followed indirectly by monitoring changes in the rate of ATP hydrolysis (Burton et al. 2003, Hall et al. 2016). The free inorganic phosphate generated during the enzymatic hydrolysis of ATP by LepClpX was quantified as described before for ClpC in *M. tuberculosis* (Akopian et al. 2012) (**Appendices A.6 A** & **B**). The LepClpX exhibited a surge in ATPase activity with increasing ATP concentration (0.25-4.0 mM), and after that, the activity reached saturation at the tested ATP concentration (4.0-8.0 mM) (**Figure 2.11 A**). The ATPase reaction follows Michaelis-Menten kinetics and agrees to an earlier study on ATP-dependent protease (Ti and ClpX) of *E. coli* (Hwang et al. 1988, Burton et al. 2003). The calculated half-maximal ATP concentration for LepClpX (K_M) of *Leptospira* was 0.78 ± 0.07 mM (**Figure 2.11 A**). Besides, we carried out a peptidase/protease assay using the substrates S1, S2, and S3 to investigate if pure LepClpX has any peptide hydrolysis activity. As expected, LepClpX did not exhibit any peptidase/protease activity on substrates S1, S2, and S3 (data not shown). These observations also advocate that there are no contaminants of *E. coli* ClpP that might have got associated during the purification of LepClpX. We next addressed whether the presence of protein substrates like casein could enhance the LepClpX ATPase activity in a fashion similar to that reported in other chaperones like LepHslU (Dong et al. 2017) and EcoClpA (Hwang et al. 1988). To our surprise, the presence of FITC-casein (S3) and β-casein (S4) substrates stimulated the ATPase activity of LepClpX by 1.8-fold compared to the control (no substrate), whereas the small peptide substrates (S1 and S2) failed to significantly stimulate the ATP hydrolysis by LepClpX (**Figure 2.11 B**). This shows that LepClpX utilizes the energy for unfolding the protein substrate (casein), as increasing the concentration of casein leads to an increase in reaction rate of ATP hydrolysis. Moreover, no such increase in ATP hydrolysis was observed in LepClpX on the addition of small peptide substrate.

Previous studies on *C. difficile* (Lavey et al. 2018) and *P. aeruginosa* (Hall et al. 2016) have described ClpP1-ClpX together form an active protease complex, and the genes encoding these components are regulated under a single operon. Similarly, the CDS of *clpP1* and *clpX* in *L. interrogans* are separated by only ten bps and apparently are regulated under a single operon. Thus, it was interesting to address if the association of LepClpX with pure LepClpP isoforms

or its mixture could stimulate protease activity on fluorogenic substrate S3. Unexpectedly, LepClpX failed to stimulate the pure LepClpP isoforms enzymatic activity in the presence or absence of ATP. However, in agreement to our peptidase assay of LepClpP isoforms (**Figure 2.4 A**), the LepClpX was able to stimulate LepClpP isoforms mixture for protease activity on fluorogenic substrate S3 in the presence of ATP (**Figure 2.11 C**). Moreover, the LepClpX in the presence of non-hydrolysable ATP-analogue (ATPγS) failed to stimulate the LepClpP isoforms mixture for protease activity (**Figure 2.11 C**). This biochemical behavior of LepClpX agrees with the previous findings where ATP hydrolysis is required for unfolding the protein substrate and permit its passage in the axial-pore of the ClpP catalytic chamber (Andreas Martin et al. 2008, Baker and Sauer 2012). It was also interesting to address if the mutation in catalytic-triad of LepClpP isoforms could affect its proteolytic activity. Therefore, the protease activity of the mutant LepClpP protease macromolecule in association with LepClpX was evaluated. However, the chaperone LepClpX could not stimulate the mutant LepClpP protease macromolecule (LepClpP1^{S98A}P2 and LepClpP1P2^{S97A}) in degrading the fluorogenic substrate S3 (**Figure 2.11 C**). Thus, the conformational orientation of all the 14-catalytic triad of LepClpP protease is the pre-requisite of controlled degradation processes in addition to the concerted action of energy-dependent chaperone LepClpX.



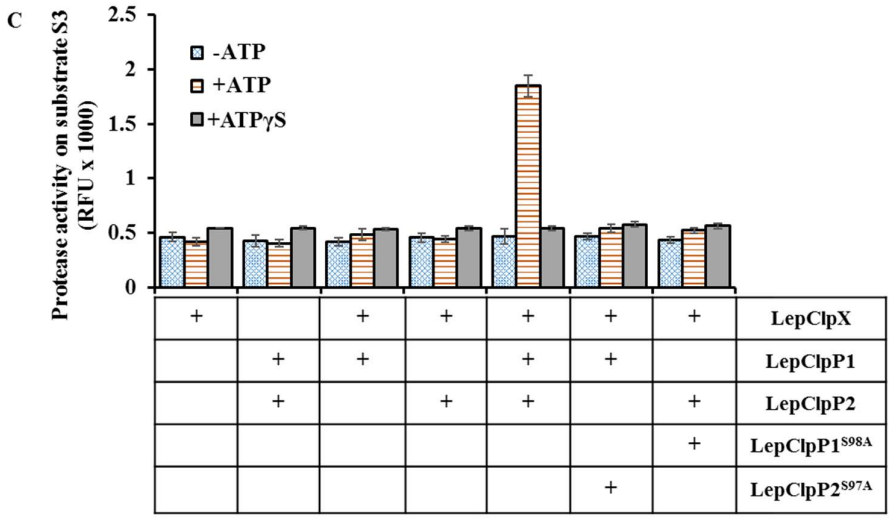


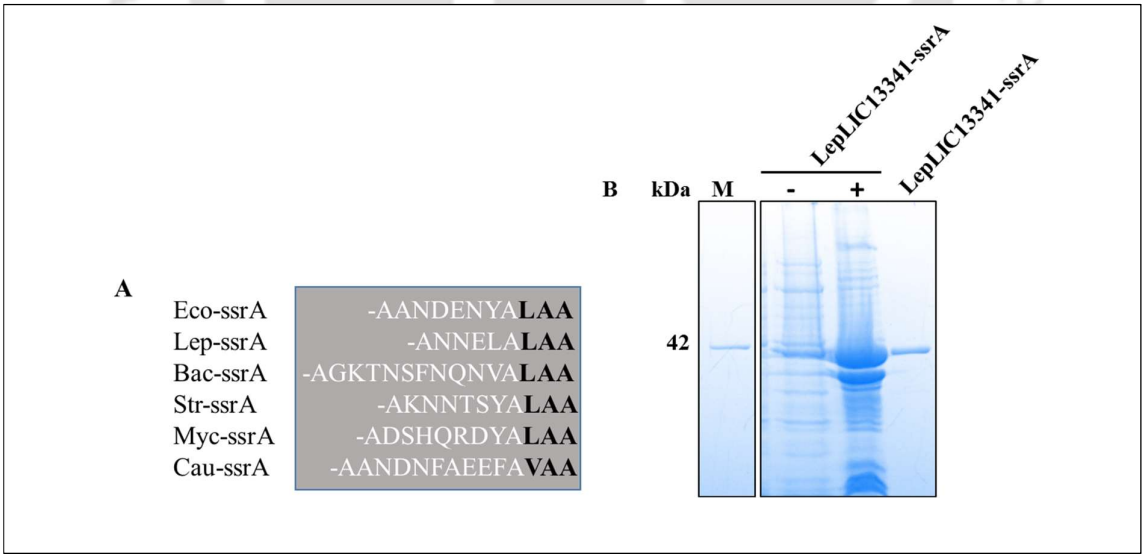
Figure 2. 11 The biochemical activity of purified recombinant LepClpX.
(A) Effect on ATPase activity of pure LepClpX at increasing concentration of ATP. The rate of ATP hydrolysis by LepClpX increases as a function of ATP concentration and gets saturated at 8 mM. The kinetic constants of the LepClpX enzymatic reaction on ATP was determined using Michealis-Menten kinetics. The K_M or the substrate concentration corresponding to the half-maximal velocity of the reaction and maximal reaction velocity during ATP hydrolysis was determined by the non-linear curve fitting function of the Origin Software. The calculated Vmax and K_M of the LepClpX was 2.63 ± 0.07 μM min^{-1} and 0.78 ± 0.07 μM, respectively. **(B)** The ATPase activity of pure LepClpX is increased in the presence of protein substrates. Increase in ATPase activity of pure LepClpX was observed in the presence of bigger protein substrates (S3 and S4) and was unaltered in the presence of small peptide substrates (S1 and S2). Substrates S3 and S4 stimulate ATPase activity of LepClpX in comparison to control where no peptide/protein substrate was present. The measured ATPase activity of LepClpX for 1 h at 37ºC (control) was considered as 100%. **(C)** Comparison of protease activity of wild-type and mutant LepClpP isoforms mixture in presence of LepClpX. The wild-type LepClpP isoforms mixture is stimulated by LepClpX to degrade fluorogenic substrate S3 only in the presence of ATP. Substitution of ATP with ATPγS, a non-hydrolysable ATP analogue, impairs substrate S3 degradation. The pure LepClpP isoforms and the mutant LepClpP isoforms mixture fails to degrade substrate S3 in the presence of LepClpX and ATP. The error bars represent the standard deviations (SDs) from the two independent experiments performed in duplicates. Statistical analysis was performed by Student's t test for comparing the LepClpX ATPase activity obtained for control and in presence of different substrates after 1.5 h of the of the assay (p-value<0.05).

2.3.8 Protease activity of LepClpXP1P2 against LepLIC13341-ssrA

We were also interested in checking the protease activity of LepClpX in complex with LepClpP isoforms mixtures against a ssrA-tagged protein substrate. The ssrA peptide tag serves as a recognition signal for proteolysis of the incompletely synthesized proteins that get modified at the C-terminus by the tmRNA rescue system in bacterial species like *E. coli, Caulobacter, B. subtilis* (Chien et al. 2007, Gottesman et al. 1998, Wiegert and Schumann 2001). *ssrA* genes are widely conserved among all bacteria, and most encode a ssrA sequence of approximately 10-11 residues (Keiler et al. 2000). In *E. coli*, this peptide tag consists of 11 residues

(AANDENYALAA) with a conserved C-terminal LAA sequence (Lies and Maurizi 2008). Like *Proteobacteria*, ssrA tags in Gram-positive bacteria are also conserved except for the last three terminal residues, LAA is sometimes replaced with VAA (Ahlawat and Morrison 2009, Wiegert and Schumann 2001). Substrates having the *E. coli* ssrA tag are degraded by EcoClpXP, with the C-terminal LAA tag residues being critical for recognition (Flynn et al. 2001). The *C. crescentus* ssrA peptide contains a similar C-terminal VAA recognition motif (Keiler et al. 2000). Similarly, by *in silic*o analysis, it is predicted that the leptospiral ssrA peptide tag consists of nine residues (ANNELALAA) with the same C-terminal recognition motif LAA (**Figure 2.12 A**). We attempted to fuse the predicted ssrA peptide sequence of *Leptospira* at the C-terminus of a leptospiral protein LepLIC13341, previously generated in our lab. The overexpressed LepLIC13341-ssrA was purified using Ni-NTA affinity column chromatography (**Figure 2.12 B**) described previously in our lab (Ghosh et al. 2018b).

Five proteases (Tsp, FtSH, Lon, ClpAP, and ClpXP) are involved in the degradation of ssrA-tagged protein in *E. coli* (Lies and Maurizi 2008, Choy et al. 2007, Herman et al. 1998, Spiers et al. 2002, Gottesman et al. 1998). Amongst them, EcoClpXP and EcoClpAP degrade most of the ssrA-tagged peptides regardless of their inherent stability (Gottesman et al. 1998, Herman et al. 1998, Weber-Ban et al. 1999, Burton et al. 2001, Lee et al. 2001). We also expected a similar result, where LepClpX could recognize the ssrA tag, and the substrate would be degraded by the LepClpP functional protease. However, surprisingly, LepClpXP1P2 could not degrade the LepLIC13341-ssrA even after prolonging the reaction for 16 h (**Figure 2. 12 D**).



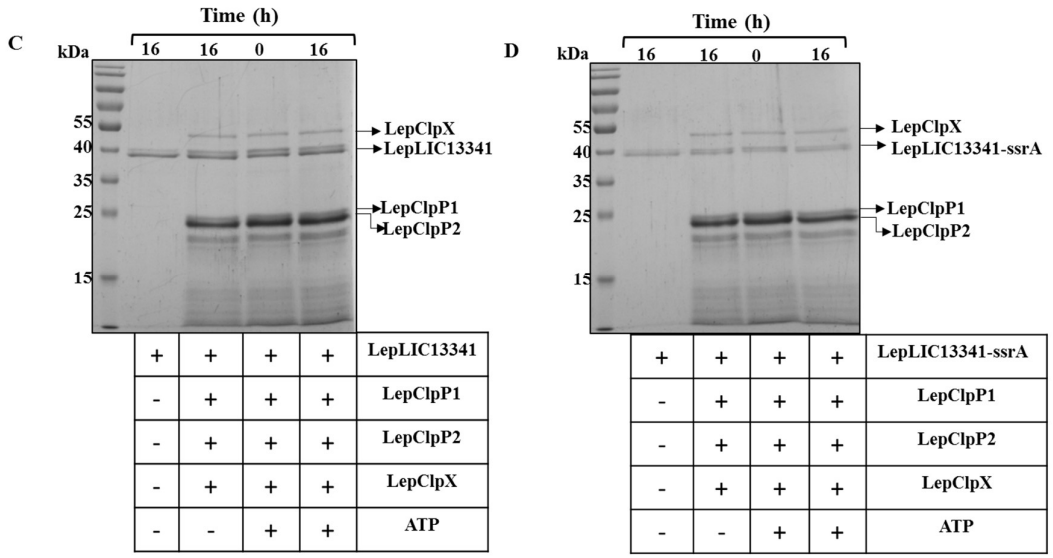


Figure 2. 12 Protease activity of LepClpXP1P2 against LepLIC13341-ssrA.
(**A**) Sequences of *E. coli* ssrA peptide tag (Eco-ssrA), *L. interrogans* ssrA peptide tag (Lep-ssrA), *B. subtilis* ssrA peptide tag (Bac-ssrA), *S. pneumoniae* ssrA peptide tag (Str-ssrA), *M. tuberculosis* ssrA peptide tag (Myc-ssrA), *C. crescentus* ssrA peptide tag (Cau-ssrA). Regions critical for ClpX recognition are highlighted. (**B**) The Coomassie-blue stained 12% SDS-polyacrylamide gel showing the resolved protein lysates of *E. coli* cells overexpressing *Leptospira* LIC13341-ssrA (pET28a-LIC13341) after induction with (+) or without (-) 1 mM IPTG and Ni-NTA affinity purified LepLIC13341-ssrA (~42 kDa). M denotes the protein marker of 42 kDa. **(C and D)** 12% SDS-polyacrylamide gel showing the reaction products of protease activity of LepClpXP1P2 against substrates LepLIC13341 and LepLIC13341-ssrA, respectively. There was no degradtion of LepLIC13341-ssrA even in the presence of LepClpX and ATP. The LepLIC13341 is taken as a control substrate for the protease reaction. Both the reactions consist of 1 µg LepClpP1P2, 0.2 µg LepClpX and 0.5 µg substrate. The reactions were carried out at 37°C. The denaturing polyacrylamide gels were stained with Coomassie-blue and for clarity, the image obtained is represented after inversion.

2.4 CONCLUSION

Exploration of genomic and proteomic data of *L. interrogans* specified that the ClpP system in *Leptospira* might exhibit a divergent behavior from that of other reported multi-ClpP organisms studied to date. We have unraveled unconventional oligomeric self-assembly of *Leptospira* ClpP1 and ClpP2 mixture intrinsically active and distinct from other ClpPs, especially the well-characterized *Mycobacterium* ClpP isoforms. Earlier reported ClpP orthologs in pathogenic bacteria demonstrated one or both the pure isoforms of ClpP could self-assemble into functional tetradecamer form whereas, in *Leptospira,* both the pure isoforms, despite self-assembling, were found to be functionally inactive. The suggested model of active peptidase complex is depicted in **Figure 2.13**.

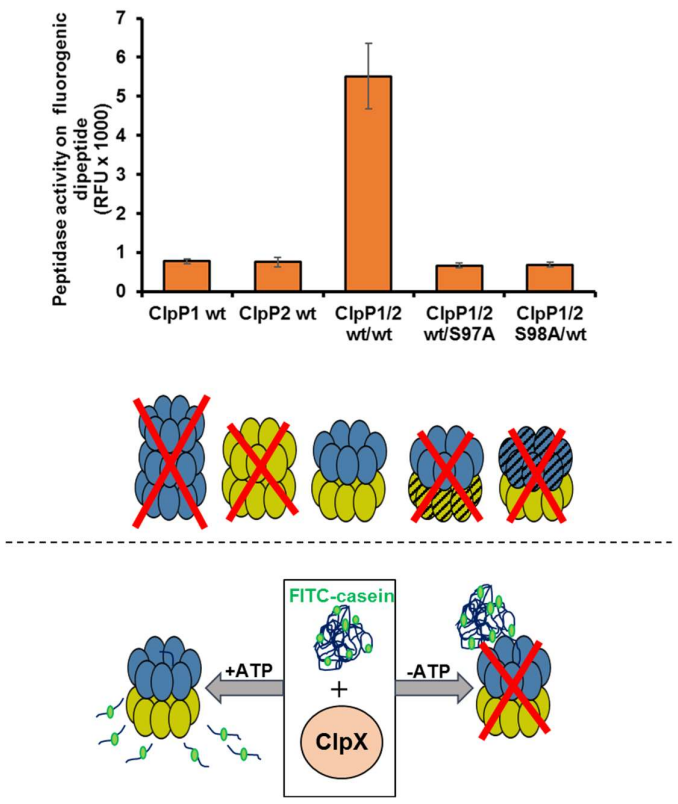


Figure 2. 13 Peptidase/protease activity of leptospiral ClpP wt and mutant isoforms.
(Upper panel) Peptidase activity of LepClpP wt and mutant isoforms on fluorogenic dipeptide substrate. Pure ClpP1 or ClpP2 isoforms are completely inactive against the dipeptide substrate, and only shows activity when both ClpP isoforms are mixed together. The leptospiral ClpP peptidase mixture ceases to operate when any one of the ClpP component is replaced with its counter serine mutant i.e. LepClpP1^{S98A} or LepClpP2^{S97A}. **(Lower panel)** Protease activity of leptospiral ClpP isoforms in presence of ClpX on FITC-casein substrate. ClpP isoforms mixture is able to degrade FITC-casein substrate exclusively in presence of ClpX and ATP. ClpX ATPase derives the energy from ATP hydrolysis to unfold the substrate and channelizes it through the axial pore of the proteolytic chamber of ClpP isoforms mixture for degradation inside the barrel-shaped ClpP protease complex harbouring the catalytic sites.

The exploitation of this unprecedented regulation of proteolytic complexes may anticipate developing a useful therapeutic target because of the broad diversity between bacterial and mammalian proteolytic systems (Ravikiran M Raju et al. 2012). However, to precisely understand the existing evolutionary invented ClpP diversity, elucidation of the catalytic triad's conformation in *Leptospira's* protease complex by high-resolution X-ray structure is further warranted. Likewise, genetic manipulation of the ClpP system in recalcitrant *L. interrogans* may throw light on the biological significance of harboring multi-isoform of ClpP and chaperone proteins. Substrate specificity has been demonstrated by the ClpP1P2 complex of *M. tuberculosis* (Akopian et al. 2012, Akopian et al. 2015). However, the substrate cleavage

preference for leptospiral pure ClpP isoforms or its mixture demands further exploration. The role of other classes of chaperones and adaptor proteins in regulating ClpP biochemical behavior in *Leptospira* remains to be answered.

2.5 MATERIALS AND METHODS

2.5.1 Bacterial strains, primers, and plasmids

Bacterial strains, primers, and plasmids used in the work are listed in **Table 2.2**. *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Lai, and *L. biflex*a serovar Patoc were obtained from Indian Council of Medical Research (ICMR), Regional Medical Research Centre (RMRC), Port Blair, Andaman and Nicobar Island, India. Spirochetes were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) media at 28-30°C at an interval of 5-7 days. Luria-Bertani (LB) medium was used for culturing *E. coli* DH5α and BL21 (DE3) (Novagen) required for cloning and expression of recombinant proteins.

Table 2. 2 Bacterial strains, plasmids, and primers used in this study

Bacterial strains, plasmids or primers		**Characteristics or Sequence**	**Source or Reference**
Bacterial strains	*L. interrogans* serovar Copenhageni strain Fiocruz L1-130	Wild-type	ICMR, Port Blair
	L. interrogans serovar Lai		
	L. biflexa serovar Patoc		
	E. coli DH5α	supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	Novagen
	E. coli BL21 (DE3)	F^- ompT hsdSb ($rBmB^-$) gal (λ c I 857 ind 1 Sam7 nin5 lacUVt7gene 1) dcm (DE3)	
Plasmids	pTZ57R/T	Linearized TA vector with 3'-ddT overhangs for TA cloning of PCR products	Thermo Scientific
	pET23a	Bacterial vector for expression of C-terminally His_6-tagged proteins	Novagen
Primers	*clpP1*F	5'CTAGCTAGCATGGCGTAATC CCGTATGTG3'(*Nhe*I)	This work
	*clpP1*R	5'CCGCTCGAGTTCAGTTTGTT TACGATCGATCT3'(*Xho*I)	
	*clpP2*F	5'CTAGCTAGCATGCCAGAAA CAGAAAAATCG3' (*Nhe*I)	
	*clpP2*R	5'CCGCTCGAGATTAAAATCG ATTTTAGTAGCGAG3' (*Xho*I)	
	*clpX*F	5'CTAGCTAGCTTGGCTAAGA AAACACCGG3' (*Nhe*I)	
	*clpX*R	5'CCGCTCGAGAGCAATCTTA GATTCTTTTTTGAG3' (*Xho*I)	
F: Forward primer, R: Reverse primer, underlined areas denote the restriction site			

2.5.2 RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the *L. interrogans* culture, grown till the log-phase, using the TRIzol (Invitrogen) method. The obtained total RNA was treated with DNase (New England BioLabs [NEB]) and converted to cDNA as described before in our laboratory (Ghosh et al. 2018a). The oligonucleotides used for qRT-PCR were designed using the available *L. interrogans* serovar Copenhageni genomic sequence as the template by the OligoPerfect primer design program (Invitrogen). The PCR (CFX96 Real-Time, Bio-rad) system was programmed for 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min, followed by melt curve analyses of the PCR products. The primers used in RT-PCR are listed in **Table A.1**. Transcripts of target genes were quantified using $2^{-\Delta\Delta C_T}$ method (Schmittgen and Livak 2008) and normalized with the 16S *rRNA* gene of *Leptospira*.

2.5.3 Cloning, expression, and purification of recombinant proteins

The full-length genes *clpP1* (*LIC11417*), *clpP2* (*LIC11951*), and *clpX* (*LIC11418*) of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 were PCR amplified using its genomic DNA as a template. The oligonucleotides for PCR were designed using the genomic sequence of *L. interrogans* Copenhageni strain Fiocruz L1-130 available on National Centre for Biotechnology Information (NCBI). The *clpP1, clpX*, and *clpP2* genes were cloned individually into pET23a vector at the *Nhe*I and *Xho*I multiple cloning sites that can express a C-terminal $(His)_6$ tagged recombinant protein. Sequencing was performed by outsourcing (Eurofins, India) the DNA to rule out any mutation in the generated recombinant pET23a plasmids. The recombinant plasmids were transformed in *E. coli* BL21 (DE3) cells and the transformed cells were cultivated at 37°C in LB medium supplemented with 100 µg mL^{-1} ampicillin. Protein expression in the transformed BL21 (DE3) cells were induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). After cultivation of the bacterial cells for another 6 h at 37°C, cells were harvested and washed with 1x phosphate buffer saline (PBS pH 7.4, 10 mM sodium phosphate, 137 mM NaCl and 2.7 mM KCl) by centrifugation at 3000 x g for 5 min to remove cellular debris. The recombinant proteins were purified by affinity column chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resins (Invitrogen). The recombinant LepClpP1 and LepClpP2 protein purification were carried out in native conditions as described before in our laboratory (Dixit et al. 2016). The recombinant proteins were eluted in Buffer-A (50 mM Tris-Cl pH 8.0, 300 mM NaCl, 250 mM imidazole) containing 10% glycerol. For recombinant ClpX purification, a hybrid method was followed, as described previously(Ghosh et al. 2018a). Briefly, *E. coli* BL21-pET23a-clpX cells expressing LepClpX

were lysed in Denaturing lysis buffer-B (200 mM sodium phosphate pH 7.8, 500 mM NaCl, 8 M urea). The soluble lysate was allowed to bind to the Ni-NTA beads and washed with Native wash buffer-C (50 mM Tris-Cl pH 8.0, 300 mM NaCl). The bound recombinant protein to beads was eluted in Native elution buffer-D (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 250 mM imidazole) containing 10% glycerol. Elutes of purified LepClpP1, LepClpP2, or LepClpX were concentrated using 10 kDa centrifugal filter units (Amicon, catalog no. UFC901024) and dialyzed against Buffer-E (50 mM Tris-Cl pH 8.0, 100 mM NaCl) or 1x PBS, both containing 10% glycerol. The purified proteins were visualized on 12% sodium dodecyl sulfate-polyacrylamide gel by Coomassie staining. Protein concentrations were estimated by Bradford method with bovine serum albumin (BSA) as standard or by measuring the absorption at 280 nm using the extinction coefficients of LepClpP1 ($\varepsilon^{0.1\%}$ = 0.741 [mg/mL]$^{-1}$ cm^{-1}), LepClpP2 ($\varepsilon^{0.1\%}$ = 0.856 [mg/mL]$^{-1}$ cm^{-1}) or LepClpX ($\varepsilon^{0.1\%}$ = 0.261 [mg/mL]$^{-1}$ cm^{-1}) calculated from the amino acid composition using program ProtParam (Kelly et al. 2005).

2.5.4. Site-directed mutagenesis of leptospiral ClpP isoforms

A site-directed single amino acid mutation was introduced in the generated recombinant plasmids pET23a-*clpP1* and pET23a-*clpP2* using Q5 site-directed mutagenesis kit (NEB, catalog no. E0554S). Using the NEBaseChanger tool, primers were designed for site-directed mutation at 98th and 97th Ser to Ala residue in LepClpP1 and LepClpP2 (**Table 2.3**), respectively, in the generation of LepClpP1^{S98A} and LepClpP2^{S97A}. The LepClpP1^{S98A} and LepClpP2^{S97A} variants were overexpressed and purified from *E. coli* cells as described for the LepClpP isoforms.

Table 2. 3 Primers used in this study for site-directed mutagenesis in the ClpP isoforms of *Leptospira*

Primer Name	Primer sequence (5'-3')	Source or Reference
clpP1(S98A)F	5'TCAGGCTTCTgctCTATGGCGGC3'	This work
clpP1(S98A)R	5'CCTAAACAAAGAGTTCTTACATC3'	This work
clpP2(S97A)F	5'AATGGCTGCTgctCTATGGGTTCTG3'	This work
clpP2(S97A)R	5'CCCATACAAACCGTGTGAAC3'	This work
F: forward primer. R: reverse primer. The lower case areas indicate the site of mutation		

2.5.5 Generation and purification of LepLIC13341-ssrA

The amino acid sequence of *Leptospira* tmRNA tag peptide (ssrA tag) was retrieved from http://www.ag.auburn.edu/mirror/tmRDB/. Based on the finding, the nucleotide sequence of

the ssrA tag was introduced at the 3'-end of *LIC13341* in the plasmid of pET28a-*LIC13341* (overexpressing LepLIC13341), previously generated in the lab, using Q5 site-directed mutagenesis kit (NEB, catalog no. E0554S). Using the NEBaseChanger tool, primers were designed to insert the peptide tag at C-terminal in the LepLIC13341 (**Table 2.4**). The LepLIC13341-ssrA variant was overexpressed and purified from *E. coli* BL21 (DE3) cells by Ni-NTA affinity chromatography.

Table 2. 4 Primers used to insert ssrA tag at the C-terminal of LepLIC13341

Primer name	DNA sequences (5'-3')	Source or Reference
LIC13341-ssrA_F	GGCGCTGGCGGCG*TGACATCATCATCATCATCAC*	This work
LIC13341-ssrA_R	AGTTCGTTGTTCGC*CTCGAGTTCTTGCTTGGAAAC*	This work
F: Forward primer; R: Reverse primer; underlined and italicized areas indicate target-specific primer to insert ssrA tag at 3'-end		

2.5.6 Generation of polyclonal antibodies against purified LepClpP1 and LepClpP2.

Antibodies against LepClpP1 were generated in rabbits by outsourcing the purified protein to Abgenex, Bhubaneswar, India. To generate anti-LepClpP2 antibody, 4-6 weeks old BALB/c mice were immunized with purified LepClpP2 subcutaneously. About 10 µg per mouse of LepClpP2 protein emulsified in Freund's complete adjuvant (FCA; catalog no. sc-3727; Santa Cruz Biotechnology) was used for primary immunization (4 mice per group). A negative control group was injected with an equal volume of PBS along with the adjuvant. Immunized mice were further given two booster injections of LepClpP2 antigen emulsified in Freund's incomplete adjuvant (FIA; catalog no. 3726; Santa Cruz Biotechnology) at 14 and 24 days of primary immunization. At 10 days after the second booster, blood was collected from each mouse by retro-orbital bleeding, and then the mouse was sacrificed using the atlantooccipital dislocation method as described previously (Ghosh et al. 2018a). Sera obtained were pooled for antibody titer analysis by enzyme-linked immunosorbent assay (ELISA) before experimental use. Immunization experiments with mice were performed at the Department of Veterinary Microbiology, College of Veterinary Science, Assam Agriculture University Guwahati, India, after the Institutional Animal Ethics Committee's approval.

2.5.7 Western blotting of *Leptospira* whole cell lysates

Whole-cell lysates of 10^9 spirochetes were resuspended in SDS loading dye to detect ClpP1 and ClpP2 expression in *Leptospira*. The resulting lysates of *Leptospira* were resolved in 12%

SDS-PAGE and transferred to a nitrocellulose membrane (Santa Cruz Biotechnology). Membranes were blocked with 5% non-fat dried milk prepared in Tris-buffered saline (TBS pH 8.0) containing 0.05% Tween 20 (TBS-T) and separately probed with anti-LepClpP1 (1:1000) or anti-LepClpP2 (1:250) antibodies for 2 h at room temperature. After being washed, the membranes were incubated with HRP-conjugated goat anti-rabbit or anti-mouse IgG (1:5,000; Sigma) for 1 h, and immunoblots were developed by adding chemiluminescence substrate (Thermo Scientific, catalog no. 32209). All dilutions of antibodies were prepared using 2% non-fat dried milk in 0.1% TBS-T.

2.5.8 Peptide hydrolysis

Peptidase activity was monitored by the rate of production of fluorescent AMC (7-amino-4-methyl coumarin) after cleavage from fluorogenic peptide substrates. The peptide substrates used in the hydrolysis assay were N-succinyl-Leu-Tyr-AMC (Suc-LY-AMC) as substrate 1 (S1) and Suc-LLVY-AMC as substrate 2 (S2) (Sigma). Peptidase assays were performed in black flat-bottom 96-well plates (Invitrogen) at 37°C. Each 96-well contained fluorogenic peptide substrate (0.1 mM) and pure LepClpP isoforms or its mixture (0.0125 μg μL^{-1}) in 80 μL of Buffer-F (50 mM phosphate buffer pH 7.6, 100 mM KCl, 5% glycerol). Fluorescence was measured in the Infinite M200Pro plate reader (Tecan) at 380 and 460 nm wavelength of excitation and emission, respectively. The peptide substrate S1 (0.01-5.0 mM) was incubated with LepClpP isoforms mixture (0.025 μg μL^{-1}) in 80 μL of buffer-F to determine the kinetic parameters of leptospiral LepClpP isoforms mixture. The subsequent experimental procedure to detect the hydrolysis of substrate S1 was the same as described above. Initial and final readings were taken at 0 h and 1 h, respectively (excitation: 380 nm/emission: 460 nm). The measurements obtained were processed in Microsoft Excel, and then data were transferred to Origin9.0 for Hill kinetics and statistical analysis. Similar peptidase assays were carried out on substrates S1 and S2 using pure LepClpP mutant isoforms (LepClpP1^{S98A} and LepClpP2^{S97A}) or the LepClpP isoform mixture constituted by either of the mutant isoforms. All the experiments were performed twice independently and in duplicates.

2.5.9 Multiple sequence alignment of ClpX orthologs and homology modeling of leptospiral ClpX

Amino acid sequences of ClpX orthologs from different pathogenic bacteria were retrieved from the UniProtKB database (Consortium 2014), and multiple sequence alignment (MSA) was performed using Clustal Omega software (Sievers et al. 2011). MSA is represented using the online tool ESPript (Easy Sequencing in PostScript) for better clarity (Robert and Gouet

2014). The secondary structures used in the study were obtained from the protein data bank (PDB) (Berman et al. 2000). Based on the sequence identities, the tertiary structure models of the ClpX *L. interrogans* serovar Copenhageni were predicted using the web-based server SWISS-MODEL (Peitsch 1996, Peitsch 1997). *Leptospira* ClpX oligomeric model was developed using the template of the known ClpX crystal structure of *L. monocytogenes* (LisClpPX; PDB id: 6sfw). All the computational ClpX structures were generated using the program PyMOL.

2.5.10 ATPase assay of *Leptospira* recombinant ClpX chaperone

The ATPase assays were carried out using ATPase/GTPase activity assay kit (Sigma, catalog no. MAK113). The amount of free phosphate determined from the standard graph was used to calculate the ATPase activity of LepClpX on ATP in µmole $min^{-1}µL^{-1}$ or µM min^{-1}. The ATP hydrolysis rates of LepClpX (0.05 µg $µL^{-1}$) were monitored with increasing concentrations of ATP (0-8 mM) in 40 µL of Buffer-G (50 mM Tris-Cl pH 7.8, 50 mM KCl, 1 mM DTT, and 8 mM $MgCl_2$) at 37°C for 1.5 h. The measurements obtained were processed in Microsoft Excel, and then data were transferred to Origin9.0 for Michaelis-Menten kinetics and statistical analysis. In addition, the ATPase activity of LepClpX (0.08 µg $µL^{-1}$) was measured in the presence of various substrates (0.1 µg $µL^{-1}$) including small peptides (S1 and S2) and large protein substrates (Fluorescein isothiocyanate tagged-casein; FITC-casein [S3] and β-casein [S4]) in a total reaction volume of 25 µL in Buffer-G at 37°C for 1.5 h. In every ATPase assay, the reaction mix was prepared with the activity buffer, LepClpX, and the substrate, pre-incubated at 37°C for 10 min before the addition of ATP. The absorbance measurements at 620 nm were performed using Multiskan GO Microplate UV-Vis spectrophotometer (Thermo Scientific). All the experiments were performed twice independently and in duplicates.

2.5.11 Estimation of LepClpX ATPase activity

The known phosphate standards (0, 12.5, 25, 31.25, 37.5, and 50 µM) were prepared from a stock 1mM Phosphate Standard provided in the ATPase activity assay kit (Cat No. MAK113). The absorbance values obtained at A620 nm of these standards were used to generate the phosphate curve. The standard curve determined the concentration of free phosphate generated by the ATPase activity of LepClpX. The ATPase activity of LepClpX was calculated as described in the protocol of the assay kit: ATPase activity of LepClpX = $(S_a \times R_v)/(S_v \times T)$, where S_a = concentration of free phosphate (µM) generated by rClpX ; R_v = reaction volume (µL); S_v = sample volume (µL) added to well; T = reaction time (min).

2.5.12 Protease assay of *Leptospira* recombinant ClpP isoforms in association with its ATPase chaperone

All protease assays were performed using the protease fluorescent detection kit (Sigma, catalog no. PF0100) as per the manufacturer's instructions. The pure LepClpP isoforms or its mixture along with the chaperone LepClpX were assayed for protease activity using fluorescent protein substrate S3 provided in the kit. Briefly, 20 µL of substrate S3 (1.5 µg μL^{-1}) was pre-warmed at 37°C and incubated with pure LepClpP isoforms or its mixture (0.02 µg μL^{-1}) and rClpX (0.04 µg μL^{-1}) in Buffer-G for 10 min before addition of 2 mM ATP/ATPγS or without ATP in a total volume of 100 µL reaction. The protease reactions were done in the dark at 37°C for 1.5 h, followed by termination of reaction with the trichloroacetic acid (0.6 N). The resulting fluorescence was recorded at 492 and 519 nm wavelength of excitation and emission, respectively. A similar proteolysis assay was carried out using the LepClpP isoforms mixture of wild-type and mutant isoforms (LepClpP1^{S98A} or LepClpP2^{S97A}). All the experiments were performed twice independently and in duplicates.

2.5.13 Protease assay of LepClpP isoforms with LepClpX using LepLIC13341-ssrA tagged substrate

The LepClpP isoforms mixture along with the chaperone LepClpX were assayed for protease activity using the generated LepLIC133341-ssrA substrate (0.5 µg) in a total reaction volume of 50 µL. In each reaction tube, the LepClpP isoforms (1 µg each) were mixed with the LepClpX (0.2 µg) and incubated for 10 min at 37°C in Buffer-G. After the incubation period, the protease reactions in each tube were initiated with/without the addition of 4 mM ATP. From the total reaction volume, a given small volume (20 µL) of the reaction was terminated at the 0^{th} and 16^{th} h of the recation, after the addition of the sample buffer and heating for 10 min at 95°C. A control protease assay of the LepClpP isoforms mixture and LepClpX containing an equivalent amount of LepLIC13341 (substrate without the ssrA tag) was included for comparison. At each of the time, the reaction products were resolved on 12% SDS-PAGE and visualized by Coomassie staining.

2.5.14 Chemical cross-linking of recombinant ClpP isoforms of *Leptospira*

The pure LepClpP isoforms or its mixture (0.2 µg μL^{-1}) were cross-linked with the glutaraldehyde solution (0.02%) in assay buffer-F in a total reaction volume of 100 µL. In the case of LepClpP isoforms mixture, each isoform (0.1 µg μL^{-1}) was mixed to yield a final concentration of 0.2 µg μL^{-1}. From the total reaction volume, a 20 µL of the cross-linking reaction was terminated at various intervals (2-15 min) by addition of 4x sample loading buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue, 100 mM DTT, and 40%

glycerol) and boiling at 95°C for 10 min. A control reaction containing pure LepClpP isoforms, or its mixture without glutaraldehyde, was prepared for comparison. The reaction products at each time point were resolved on 10% SDS-PAGE and visualized by Coomassie staining.

2.5.15 Isothermal Titration Calorimetry (ITC)

ITC experiments were performed on a MicroCal iTC200 system (GE Healthcare) at 37°C in buffer-E with constant stirring at 250 rpm. The pure LepClpP1 and LepClpP2 were concentrated using 3 kDa cut-off centrifugal filter units (Amicon, catalog no. UFC800324) in buffer-E to equilibrate the buffer conditions of ITC syringe and sample solutions. The sample cell and the syringe were washed twice with buffer-E before loading the analytes. The equilibrated pure LepClpP1 (60 µL of 90 µM) and the LepClpP2 (300 µL of 9 µM) was loaded into the ITC syringe and the sample cell, respectively. The experiment was initiated after equilibration for 300 s with the first injection of LepClpP1 (0.4 µL) discarded during the analysis. The LepClpP1 (1.5 µL) was titrated into the sample cell at an interval of 120 s. Power was recorded at 'high' gain setting with a reference power of 10 µcal s^{-1} and a 5 s filter period. Data analysis, including baseline correction and evaluation, was carried out using OriginPro 8.5 ITC.

2.5.16 Size exclusion chromatography (SEC)

Enrich SEC 650 high-resolution column (10 mm x 300 mm, catalog no. 7801650) was used for SEC on an NGC™ chromatography system (Bio-Rad). The column was equilibrated with a buffer containing 50 mM Tris-Cl pH 8.0, 100 mM NaCl and 10 % glycerol. The column was calibrated with β-amylase (200 kDa), alcohol dehydrogenase (158 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome C (12.4 kDa) (Sigma, catalog no. MWGF-200). The LepClpP isoforms were used at a final concentration of 0.5 mg mL^{-1} and were incubated overnight at 4°C. The LepClpP isoform samples (250 µL) equilibrated in the same buffer were loaded onto the column, and gel-filtration experiments were carried out with a flow rate of 0.3 mL min^{-1} at room temperature.

2.5.17 Native polyacrylamide gel electrophoresis (Native-PAGE).

Pure LepClpP isoforms (2 µg) and its mixture in buffer-E were incubated at 37°C for 10 mins followed by mixing with 3x native sample buffer (240 mM Tris-HCl pH 6.8, 30% glycerol, 0.03% bromophenol blue) (Preissler et al. 2015). The LepClpP isoform subunits interaction in solution were analyzed on a 4-20% gradient gel (Bio-Rad MiniProtean, catalog no. 456-1096) after resolving for 2 h at 120 V. The resolved proteins in gradient gel were visualized using

Coomassie stain and compared with known molecular weight standard protein markers (Invitrogen, cata1og no.928387).

2.5.18 Dynamic Light Scattering (DLS)

DLS experiments were performed on a Zetasizer Nano ZS (Malvern Instruments) at 25°C. Pure LepClpP isoforms or their mixture (0.5 mg mL^{-1}) in buffer-E were incubated for 1 h or 24 h at 4°C and were added to polystyrene cuvettes. The scattering was recorded at 173° angle; a 633 nm He–Ne laser was used as the light source. Ten autocorrelation functions were recorded for each of the protein samples, and intensity-weighted hydrodynamic diameters were determined. The profile corresponding to average particle sizes was generated as reported and discussed elsewhere (LaBreck et al. 2017).

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Chapter 3

Acyldepsipeptide activated ClpP1P2 macromolecule of Leptospira, an ideal Achilles' heel to deregulate proteostasis and hamper the cell survival[2]

3.1 ABSTRACT

Antibiotic acyldepsipeptide (ADEP) targets the bacterial ClpP serine protease and can inhibit numerous bacterial species' growth by activating/dysregulating the cell's protease activity. The spirochete *L. interrogans* harbors two ClpP isoforms (LepClpP1 and LepClpP2), when grown under *in vitro* condition in the presence of ADEP, showed inhibition in growth accompanied by elongated cell morphology. The chemoactivation of the LepClpP mixture by ADEP was dependent on the self-assembly duration of LepClpP1 and LepClpP2 under *in vitro* experimental condition. In the presence of the ADEP, the change in the structural diameter of the LepClpP1P2 or its catalytic serine mutant variants using dynamic light scattering indicates a conformational transformation in the LepClpP machinery. Serine 98, a catalytic triad residue of the LepClpP1 in the LepClpP1P2 heterocomplex, is critical for the ADEP mediated activation. Chemoactivation also switches the LepClpP1P2 peptidase or LepClpXP1P2 complex into a dysregulated proteolytic machinery suggesting the toxicity of ADEP during spirochetes growth may be due to unregulated degradation of critical proteins within the cell. The computational prototype of the LepClpP1P2 structure suggests that the hydrophobic pockets wherein the ADEPs or the physiological chaperone ClpX predominantly dock are exclusively present in the LepClpP2 heptamer. Using the ADEP as a tool, this investigation provides an insight into the molecular function of the LepClpP1P2 in a coalition with its ATPase chaperone LepClpX. The shreds of the evidence illustrated in this investigation verify that the ADEP can control the LepClpP system in a different approach.

[2] This chapter is adapted from a manuscript under review. The authors are Anusua Dhara, Md Saddam Hussain, Shankar Prasad Kanaujia, and Manish Kumar. MK conceived and supervised the study; MK and AD designed experiments and analyzed the data; SPK performed docking and modeling experiments; MSH performed the growth assays and the FESEM; MK, AD, and SPK wrote the manuscript.

3.2 INTRODUCTION

Leptospira interrogans is the causative agent of leptospirosis, a globally important zoonotic disease (Bharti et al. 2003). The transmission of the pathogenic *Leptospira* between animals, humans, and the environment is essential for maintaining its enzootic cycle (Ellis 2015). Over a million leptospirosis cases are reported every year, with approximately 60000 deaths in humans (Costa et al. 2015). Leptospirosis is a zoonotic disease that disables livestock production in developing tropical and sub-tropical countries where animal rearing is a primary source of livelihood (Shiokawa et al. 2019). Antibiotics, particularly the penicillin group, are considered first-line therapy for leptospirosis (Charan et al. 2013). However, due to the emergent multi-drug resistance of the Gram-negative and Gram-positive bacteria, an urgent need for therapeutics acting on novel pathways to curtail such persistent bacteria is the need of the hour (Brunel and Guery 2017). The subcellular pathways central to the bacteria's survival during the infection are attractive candidates for new drug design. In such an effort, the acyldepsipeptides (ADEPs), a new class of antibacterial compound and its derivative were found to target the caseinolytic protease (ClpP protease), the proteolytic core of bacterial ATP-dependent proteases (Brotz-Oesterhelt et al. 2005, Gil and Paredes-Sabja 2016). ADEP1 is a natural molecule of the acyldepsipeptide family produced by *Streptomyces hawaiiensis* that function by dysregulating/activating the ClpP in other microbes, unlike other conventional antibiotics (Michel and Kastner 1985, Brotz-Oesterhelt et al. 2005, Thomy et al. 2019). Activation of the ClpP results in cell division inhibition, imbalance in cellular proteostasis, and finally, cell death of the bacteria, including *Staphylococcus*, *Streptococcus*, *Mycobacterium* (Sass et al. 2011, Famulla et al. 2016). Also, prokaryote ClpP has been found to have a crucial role in regulating processes such as stress tolerance, virulence, morphological differentiation, and antibiotic resistance (Frees et al. 2003, Frees et al. 2014, Viala and Mazodier 2003, Ingmer and Brøndsted 2009, Frees et al. 2007, Thomy et al. 2019). Dysregulating the Clp protease activity in the pathogenic bacteria by the ADEP's or other activators leads to a reduction of its chance for cell survival. The exploitation of such targets is now helpful to destroy multi-drug resistance or the persister form of bacteria emerging due to the improper use of antibiotics (Qin et al. 2018, Frees et al. 2014, Thomy et al. 2019, Conlon et al. 2013).

The caseinolytic protease system in prokaryotes is composed of the core ClpP catalytic components, regulatory chaperones (ATPases), and the adaptor protein (Olivares et al. 2018, Dhara et al. 2019). Most bacterial species, including *E. coli*, *Bacillus subtilis,* and *Staphylococcus aureus* have one *clpP* gene that, along with their associated ATPases, are nonessential for cell viability whereas, in actinobacteria and cyanobacteria, two or more copies

of *clpP* are found, and at least one functional copy is indispensable for viability (Raju et al. 2012, Gominet et al. 2011). In *E. coli,* the core catalytic component ClpP is a tetradecameric barrel-shaped serine peptidase with the 14 active sites contained within its proteolytic chamber (Wang et al. 1997). In *Mycobacterium tuberculosis*, *clpP1* and *clpP2* form an operon, and both the genes product are critical to compose an operative peptidase by stacking the MycClpP1 and the MycClpP2 homoheptamers into a heterotetradecamer (Raju et al. 2012). It is demonstrated that in *E. coli,* the core EcoClpP independently can degrade smaller peptides; however, it needs to associate with its cognate Clp/Hsp100 chaperone (Clp-ATPase) to degrade the larger polypeptides and proteins (Barkow et al. 2009). The cognate chaperones coordinate with the ClpP in substrate recognition, unfolding the substrate using energy derived from the ATP hydrolysis and delivering the unfolded polypeptide into a proteolytic compartment of the ClpP (Malik and Brötz-Oesterhelt 2017). The chaperone ClpX self-composes into a hexamer and employs its peptide loops (IGF/L) to anchor into the apical site (hydrophobic pocket) of the ClpP tetradecamer and render the opening of the entrance pore to foster access of larger substrates in a coordinated strategy (Kim and Kim 2003). It has been ascertained that in bacteria with a single ClpP isoform, a total of two ClpX or ClpA hexamers can bind to one ClpP barrel from both sites, resulting in a ClpX-ClpP-ClpX or ClpA-ClpP-ClpA complex formation (Beuron et al. 1998, Grimaud et al. 1998). Whereas, in bacteria like the *Mycobacterium*, *Listeria,* and *Chlamydia* with the multi-ClpP isoforms, the cognate ATPase chaperone has been documented to dock exclusively to the ClpP2 hydrophobic pocket (Leodolter et al. 2015, Schmitz et al. 2014, Carroll et al. 2011, Sassetti et al. 2003, Gatsogiannis et al. 2019). Biochemical studies in the *B. subtilis* infer that antibiotic ADEP1 mimics ClpX peptide loops, thereby broadening the BacClpP protease's entrance pores and could degrade larger polypeptides unaided as an unregulated protease in the absence of any unfoldase (Kirstein et al. 2009, Lee et al. 2010). In addition to the widening of the proteolytic compartment's entrance pores, ADEP stabilizes the ClpP tetradecamer and stimulates the catalysis allosterically (Gersch et al. 2015, Lee et al. 2010).

The Clp protease of the bacteria in association with the ATPase chaperone/unfoldase is a physiological prerequisite for the quality control of the cytosolic proteins (Kress et al. 2009). Manipulating the Clp protease (ClpP) function has exhibited an impact on the virulence and infectivity of several different pathogens, as discussed in an elegant review elsewhere (Moreno-Cinos et al. 2019). During the late 90s and early 21st century, the ClpP and its allied chaperones were established to have a direct connection with the virulence or stress in the *Staphylococcus aureus* (Mei et al. 1997, Frees et al. 2003, Farrand et al. 2013), *Streptococcus pneumoniae*

(Robertson et al. 2002, Kwon et al. 2004, Park et al. 2010), *Listeria monocytogenes* (Gaillot et al. 2000, Gaillot et al. 2001, Beauregard et al. 1997) and *Salmonella typhimurium* (Hensel et al. 1995, Yamamoto et al. 2001, Knudsen et al. 2013). In a later term, the functional role of the ClpP was determined in a few other microbes like *Pseudomonas aeruginosa* (Qiu et al. 2008, Bishop et al. 2017), *Legionella pneumophila* (Zhao et al. 2016), and *Chlamydia* (Wood et al. 2019, Pan et al. 2019).

To date, a significant investigation of the ADEPs has been restricted in the bacterial ClpPs in the phylum of firmicutes, actinobacteria, or chlamydiae (Ye et al. 2017, Carney et al. 2014, Malik and Brötz-Oesterhelt 2017). In spirochetes, a phylum that encompasses a catalog of pathogenic bacteria like *Leptospira*, *Borrelia,* and *Treponema*, the influence of the ADEPs on its ClpP is yet to be unveiled. In *Leptospira*, the core ClpP catalytic element exists in two isoforms, ClpP1 (LIC11417) and ClpP2 (LIC11951), which are transcribed independently (Dhara et al. 2019). In the same analysis, the *Leptospira* ClpP1 was ascertained to self-assemble into a larger molecule (14-21mer) than the pure ClpP2 (14-mer), and both of the pure isoforms were not functional (Dhara et al. 2019). Nonetheless, the two ClpP isoforms of the *Leptospira* jointly self-assembles into a heterotetradecamer structure composed of two stacked homoheptamer of the ClpP1 and ClpP2 to constitute operative peptidase machinery (Dhara et al. 2019). Therefore in this investigation, we explored the influence of the antibiotic ADEP1 on the live *Leptospira* carrying the operative LepClpP target by a bacterial growth inhibition assay. The inhibitory impact of ADEP1 on the *Leptospira* growth is validated by various *in vitro* biochemical reactions of recombinant ClpP proteins on model substrates and the computational modeling.

3.3 RESULTS

3.3.1 ADEP1 treatment elongated *Leptospira* and hampered its growth kinetics.

We examined the effect of antibiotic ADEP1 on the pathogenic *Leptospira's* growth and morphology for a period of 120 h under *in vitro* condition. The measured length of the bacteria under the microscope appeared slightly elongated within the 24 h of sub-culturing the leptospires in a media supplemented with the 10 μg mL^{-1} ADEP1 (15 μM) (**Figure 3.1 A**). The average length of the *Leptospira* cells treated with the ADEP1 ranged from 12.8-14.6 μm, whereas the untreated cells were 10.9-11.7 μm. In the presence of ADEP1 (15 μM), there was a 1.2-fold increase in the length of the leptospires. During the ADEP1 (15 μM) treatment period (24 - 120 h), the measured increase in the bacterium's length was nearly identical under the given *in vitro* condition. The growth kinetics measurement assessed the effective bactericidal

concentration of ADEP1 for *Leptospira* in the presence of the increasing concentration of ADEP1 (20 - 60 µM). As compared to untreated cells, the growth kinetics of the ADEP1 treated spirochete curtailed from 48 h onwards in proportion to the amount of ADEP1 (**Figure 3.1 B**). The decline phase of the spirochete growth curve was achieved within the 48 h of adding 43 µg mL^{-1} ADEP1 (60 µM). The spirochetes' morphology grown in the presence of a higher ADEP1 concentration (60 µM) was evaluated under electron microscopy and compared with the untreated ones under *in vitro* condition (**Figure 3.1 C**). The average length of a segment (per three complete spiral turns) of the spirochete treated with ADEP1 was longer (1.2 fold) than the untreated one, implying a reduction in spiral frequency as the rationale behind the spirochete elongation. To understand the toxic effect of ADEP1 on *Leptospira*, we examined the effect of ADEP1 on its target protein ClpP by conducting various *in vitro* biochemical analysis using the recombinant functional protein and computational modeling.

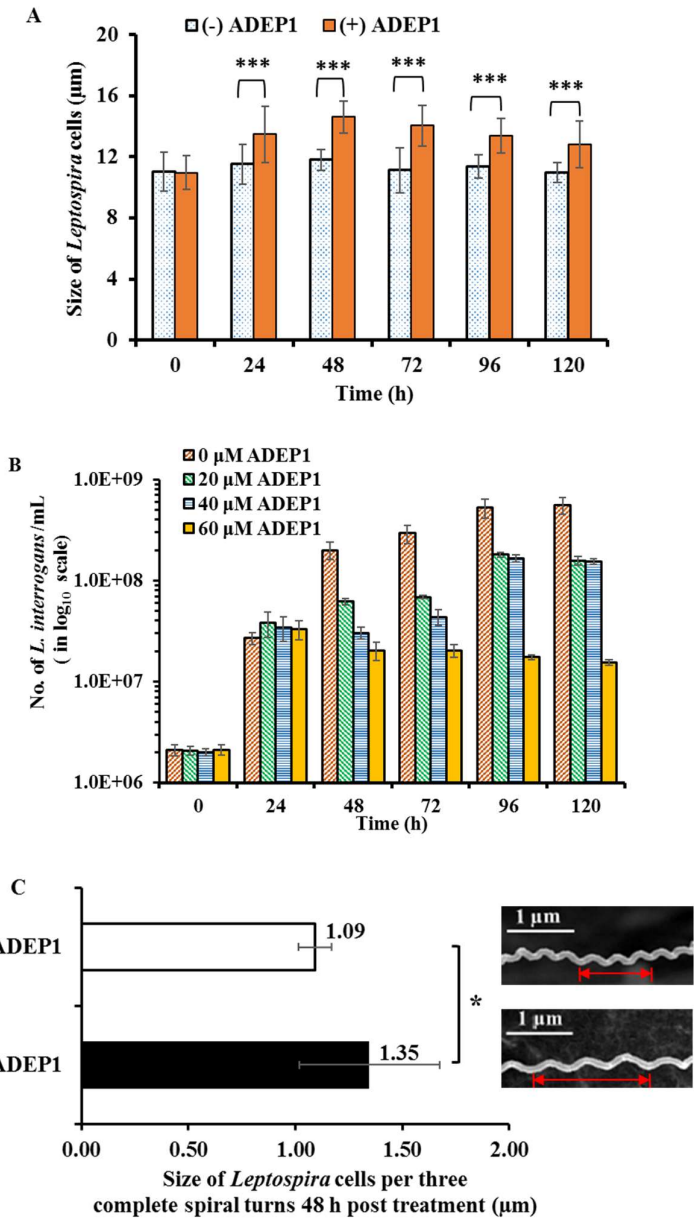


Figure 3. 1 The growth curve and the length of the pathogenic *Leptospira* are affected when grown in the presence of the ADEP1 under *in vitro* condition.

(A) Bar graph demonstrating the morphology of the *Leptospira* cells was elongated when grown in the presence of the ADEP1 after 24 h of the growth curve. **(B)** Inhibition of the growth curve of the *Leptospira* in the presence of an increasing concentration of the ADEP1 under the *in vitro* condition. The growth of the *Leptospira* was inhibited in the presence of the ADEP1 at 48 h compared to the control. Each experiment was performed independently twice with the three replicates in each set. **(C)** Field emission scanning electron microscopy to measure the morphology change of the *Leptospira* grown in the presence of the ADEP1 under the *in vitro* condition. The average partial length (three complete spiral turns) of the *Leptospira* in the presence (+) of the ADEP1 (60 µM) at the 48 h measured under the FESEM is graphically represented. The representative magnified *Leptospira* image (25 kx resolution and 1 µm scale) treated (+) with the ADEP1 appears to be more relaxed than the untreated one, with an estimated 1.23-fold elongation.

3.3.2 ADEP1 bound ClpP1P2 of *Leptospira* triggers autoproteolysis under *in vitro* condition

In our recent study, we demonstrated that the LepClpP1P2 peptidase activity is conditional on the ClpP self-assembly duration (Dhara et al. 2019). The absolute peptidase activity of LepClpP1P2 generated after a short-preincubation (1 h) for self-assembly was lower than that of long-preincubated (24 h) LepClpP1P2 (Dhara et al. 2019). The explanation for such difference in activity was apparent through the native-PAGE and the dynamic light scattering (DLS) analysis, wherein a more stable and functional population of the LepClpP1P2 tetradecamer complex was formed after the long-preincubation. In this study, we assessed the peptidase activity of the pure LepClpP isoforms or their mixture (LepClpP1P2) in the presence and absence of the ADEP1 (5 - 40 μM) towards the model fluorogenic dipeptide substrate S1 (Suc-LY-AMC) used elsewhere (Thompson and Maurizi 1994, Dhara et al. 2019). We generated the LepClpP1P2 heterocomplex by mixing the pure LepClpP isoforms under the short- (10 min) and the long-preincubation period (24 h) before assessing the peptidase activity of the operative heterocomplex in the presence of ADEP1. There was a 7-fold inhibition in the peptidase activity of the LepClpP1P2 (short-preincubated) in the presence of the ADEP1 (40 μM) compared to the basal activity without the ADEP1 (**Figure 3.2 A**). In contrast, the peptidase activity of the LepClpP1P2 (long-preincubated) got stimulated by 2.5-fold in the presence of the ADEP1 (15 - 40 μM) than its basal activity without the ADEP1 (**Figure 3.2 A**). The absolute peptidase activity of the LepClpP1P2 (short-preincubated) in the presence or absence of the ADEP1 was lower than that of the LepClpP1P2 (short-preincubated) (**Appendices B.1 A, B.1 B,** and **B.1 C**). Notably, the relative decline in the LepClpP1P2 (long-preincubated) peptidase activity was observed at the 20 - 40 μM of ADEP1 than that of the optimal 15 μM ADEP1 concentration. ADEP1 (up to 40 μM), on the other hand, failed to stimulate any peptidase activity in the pure LepClpP isoforms (data not shown). The antibiotic ADEP1 is known to activate the peptidase activity of the ClpP1P2 heterocomplex by an increase in the diameter for the substrate entry into the peptidase machinery. To comprehend the unusual effect of the ADEP1 on the LepClpP1P2 (short-preincubated), we resolved the reaction products of the peptidase reaction after completion on a denatured polyacrylamide gel. The staining of the polyacrylamide gel illustrated self-degradation of the LepClpP1P2 (short-preincubated) subunits in proportion to the ADEP1 supplemented for the peptidase activation (**Figure 3.2 B, upper panel**). In comparison, the pure LepClpP isoforms did not show any degradation in the presence of the ADEP1 (**Figure 3.2 B, middle and lower panel**). Incredibly, on a polyacrylamide gel, the LepClpP1P2 (long-preincubated) reaction product demonstrated

even more self-degradation of the LepClpP subunits (**Figure 3.2 C**). Such self-degradation of the LepClpP1P2 (long-preincubated) was contradicting to the gain in the peptidase activity of the LepClpP1P2 (long-incubated) in the presence of the ADEP1 (**Figure 3.2 A**) and motivated us to look forward to other conceivable means of chemoactivation. Hence, ADEP1 mediated functional gain in the LepClpP1P2 triggers autoproteolysis of the LepClpP protomers, and the chemoactivation of the LepClpP1P2 is dependent on the duration self-compartmentalization process of the ClpP isoforms under the given *in vitro* condition.

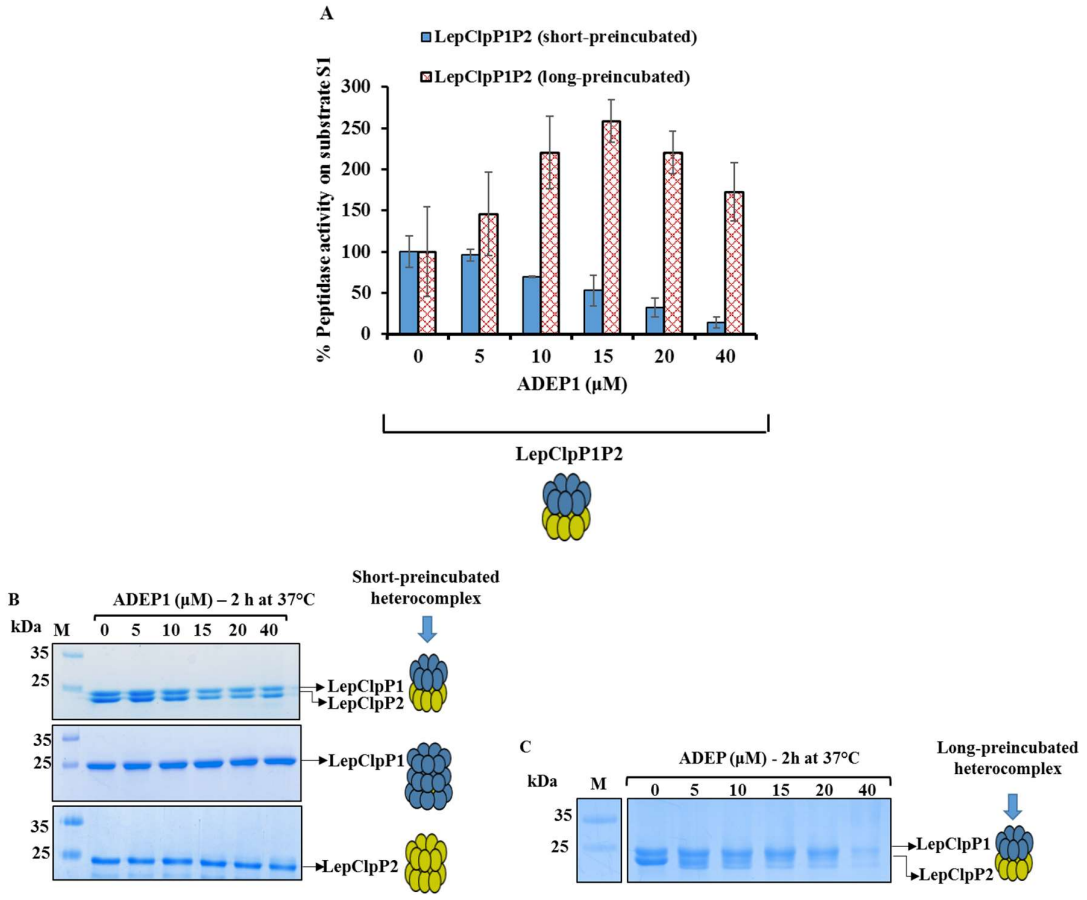


Figure 3. 2. Stimulation of the peptidase activity of the recombinant ClpP1P2 (LepClpP1P2) heterocomplex in the presence of the ADEP1 triggers auto proteolysis.
(A) Peptidase activity of the short- (10 min) and the long-preincubated (24 h) LepClpP1P2 on the small fluorogenic peptide substrate in the presence of the ADEP1. Peptidase activity of the LepClpP1P2 is represented as a percentage (%), wherein the end-point fluorescence was measured after the 2 h of the enzymatic reaction. The measured end-point fluorescence value of the LepClpP1P2 (containing no ADEP1) as control was considered 100% for measuring the relative peptidase activity. The error bars represent the standard errors of the mean (SEM) from the two-independent experiments performed. **(B)** Denaturing polyacrylamide gel electrophoresis of the pure LepClpP isoforms and their heterocomplex, LepClpP1P2 after ADEP1 treatment. **(C)** Denaturing polyacrylamide gel electrophoresis of the LepClpP1P2 (long-preincubated) after the ADEP1 treatment. For clarity, the schematics of LepClpP1 and LepClpP2 are represented where each blue and yellow sphere depicts a monomeric subunit of LepClpP1 and LepClpP2, respectively.

3.3.3 ADEP1 increases the peptidase activity of LepClpP1P2 (short-preincubated) in the presence of casein or bovine serum albumin and switch the LepClpP1P2 to ATPase independent proteolytic machinery

Within bacteria, under the natural conditions, it is unrealistic to develop conditions with a paucity of the protein substrates to the ADEP1 activated ClpP peptidase machinery. Thus, to mimic the bacteria's natural subcellular ambiance where no dearth of the substrates/proteins for the activated ClpP exists, we modified the peptidase activity assay strategy by introducing an additional β-casein (unstructured substrate) or BSA (bovine serum albumin) protein. On supplementation of the β-casein substrate, the relative peptidase activity of the ADEP1 (15 μM) bound LepClpP1P2 (short-preincubated) towards the model dipeptide substrate S1 (Suc-LY-AMC) got enhanced by a 2.6-fold than its basal level activity without ADEP1 (**Figure 3.3 A**). Similarly, on supplementation of bovine serum albumin (BSA) protein, enhancement in the peptidase activity of the ADEP1 bound LepClpP1P2 (short-preincubated) was detected; at the same time, neither the BSA nor its LepClpP protomers degradation was noticed (**Appendices B.2 A** and **B.2 B**). In contrast, in the absence of the β-casein, a 7-fold reduction in the peptidase activity by the ADEP1 (40 μM) bound LepClpP1P2 (short-preincubated) was observed relative to its basal level activity without the ADEP1 (**Figure 3.3 A**). On the other hand, the addition of ADEP1 (15 μM) to the pure LepClpP isoforms failed to display any peptidase activity in the presence or absence of the β-casein (**Figure 3.3 B**). Moreover, the β-casein substrate's addition does not lead to a change in the measured peptidase activity in the absence of the ADEP1 (**Figure 3.3 B**). The peptidase activity of the LepClpP1P2 (long-preincubated) bound to the ADEP1 demonstrated enhancement of the activity while additional supplementation of the β-casein or BSA did not lead to any further gain in its activity (**Appendices B.3 A** and **B.3 B**). The supplementation of the β-casein to the ADEP1 activated LepClpP1P2 (long-preincubated) peptidase reaction abolished the self-cleavage of the LepClpP protomers (**Appendix B.3 C**). Additionally, we also examined if the ADEP1 bound LepClpP1P2 (short-preincubated) could perform the caseinolytic activity in a chaperone-independent process. Assessment of the LepClpP caseinolytic reaction product on the denaturing polyacrylamide gel in the absence of the ADEP1 demonstrates that neither the pure LepClpP isoforms nor their heterocomplex were able to degrade the β-casein or itself (**Figure 3.4 A, 3.4 B,** and **3.4 C, upper panels**). Likewise, the presence of ADEP1 failed to demonstrate any caseinolytic or self-cleavage activity in the pure LepClpP isoforms (**Figure 3.4 A** and **3.4 B, lower panels**). However, the ADEP1 could trigger degradation of the β-casein (Figure 3.4C, lower panel) and the FITC-casein substrate (**Figure 3.4 D**) by the LepClpP1P2 (short-

preincubated) independent of the ATPase chaperone and without undergoing any self-cleavage.

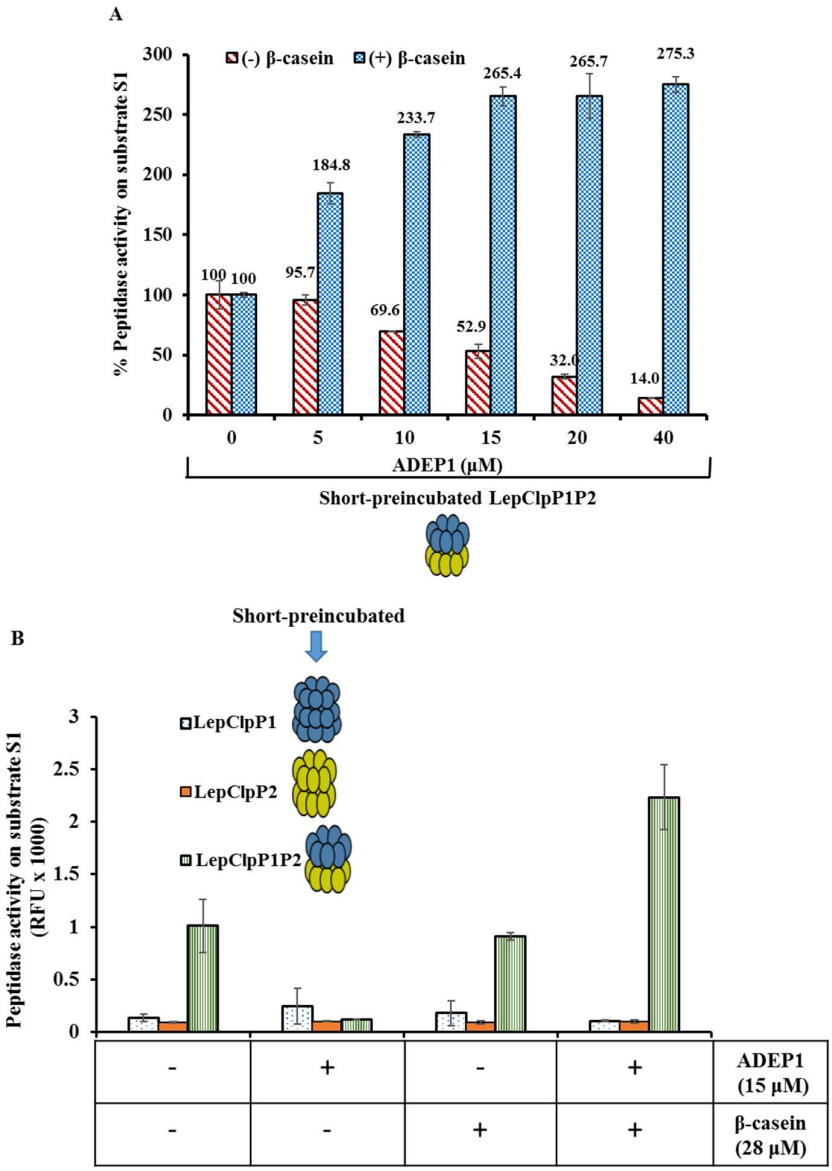


Figure 3. 3. ADEP1 stimulated the peptidase activity of the LepClpP1P2 (short-preincubated) in the presence of the β-casein.
(A) Comparison of peptidase activity of the LepClpP1P2 stimulated by ADEP1 in the presence (+) or absence (-) of excess β-casein. Peptidase activity of the LepClpP1P2 is represented as a percentage (%), wherein the end-point fluorescence was measured after 2 h of the enzymatic reaction. The measured end-point fluorescence value of the LepClpP1P2 (containing no ADEP1) as control was considered 100% for measuring the relative peptidase activity. For clarity, the schematics of the tetradecamer is shown. (B) Effect of the ADEP1 stimulated peptidase activity of the pure LepClpP isoforms and its heterocomplex LepClpP1P2 in the presence and absence of the β-casein. The error bars represent the standard errors of the mean (SEM) from the two-independent experiments performed.

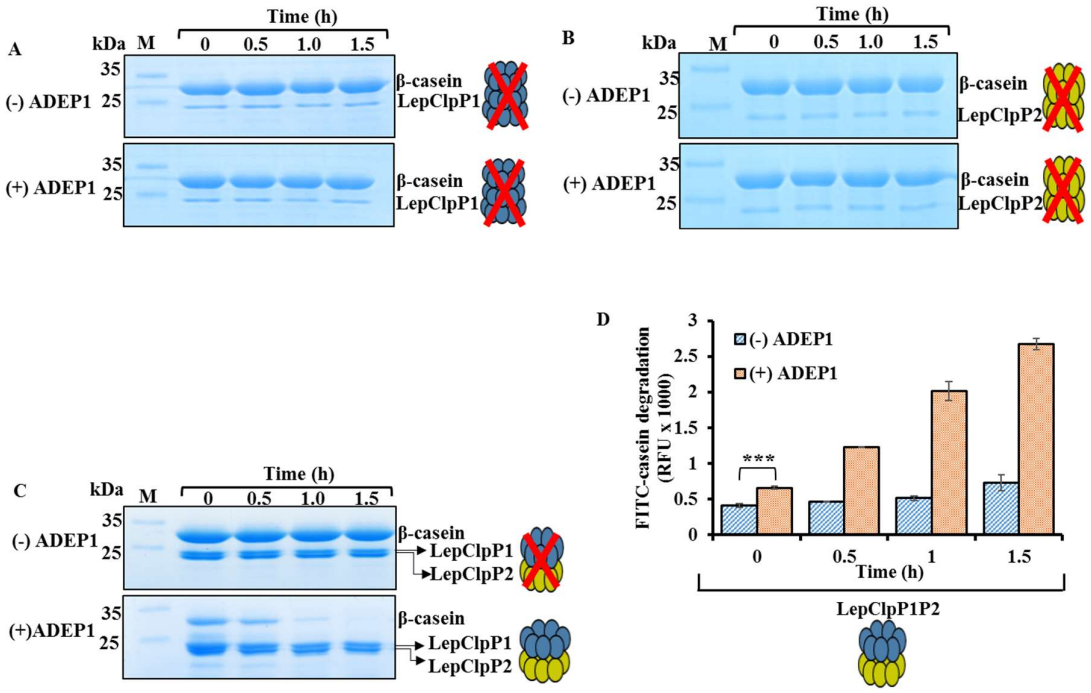


Figure 3. 4. Effect of the ADEP1 on the protease activity of the pure LepClpP isoforms and the LepClpP1P2 heterocomplex.

(A and B). Denaturing gel electrophoresis showing the activity of the pure LepClpP isoforms on the β-casein substrate in the presence (+) or absence (-) of the ADEP1. ADEP1 does not stimulate the pure LepClpP isoforms (schematics in a red cross) to degrade β-casein. **(C)** Denaturing gel electrophoresis showing the activity of the LepClpP1P2 on β-casein in the (+) or (-) of ADEP1. The red cross over the schematics of the LepClpP1P2 represents inactive heterocomplex. **(D)** Protease activity of LepClpP1P2 after stimulation by the ADEP1 on the fluorogenic FITC-casein substrate. The error bars represent the standard errors of the mean (SEM) from the two-independent experiments performed (***p-value<0.001).

3.3.4 ADEP1 exerts conformational influence over the whole tetradecameric LepClpP1P2

Functional ClpP orthologs is a highly dynamic macromolecule (Liu et al. 2014, Famulla et al. 2016), and the proposed model states that the ClpP tetradecamer, once stabilized, remains in an equilibrium between an active (extended) and inactive (compressed) state (Malik and Brötz-Oesterhelt 2017). However, the ADEP1 bound ClpP orthologs smartly switches this equilibrium towards the extended state via conformational influence (Malik and Brötz-Oesterhelt 2017). So, we have strived to validate the compressed-to-extended state transition of the ADEP1 bound LepClpP1P2 or the independent form of the tetradecamer by measuring the hydrodynamic diameter (D_h) of the tetradecamer in a solution using the dynamic light scattering (DLS) technique as suggested before for the *Thermus thermophilus* ClpP (TtClpP) (Felix et al. 2019) and SaClpP (Gersch et al. 2015). The measured diameter (D_h) of the LepClpP1P2 in the presence (15 µM) and the absence of the ADEP1 were 16.76 and 13.60 nm,

respectively (**Figure 3.5 A** and **3.5 B**). The boost in the diameter of the LepClpP machinery in the presence of the ADEP1 suggests an "open-gate" ClpP model that facilitates the entry of the unfolded substrates in the absence of the ATPase chaperone.

Our earlier study revealed that the mutant heterocomplex (LepClpP1^{S98A}P2 and LepClpP1P2^{S97A}), where one of the catalytic triad serine (98/97) residue mutation in the either of or both of the LepClpP isoforms ushers to a loss of the peptidase activity. Moreover, the active site mutant variants of either isoform of the LepClpP heterocomplex combined with its chaperone LepClpX did not show any caseinolytic activity (Dhara et al. 2019). The LepClpP active site mutant variants' biochemical activity in association with the LepClpX implies only an open-gate model of the LepClpP is not self-sustaining to gain the caseinolytic activity.

It is established that the activation of the ClpP due to the binding of the ADEP1 relays various conformational changes in the whole ClpP machinery (Malik and Brötz-Oesterhelt 2017). This motivated us to address whether the ADEP1 binding to LepClpP1^{S98A}P2 and LepClpP1P2^{S97A} steers to a perpetual increase in the D_h as scaled for the LepClpP1P2? Forth DLS analysis, the measured structural diameter (D_h) of the LepClpP1P2^{S97A} in the presence (15 µM) and the absence of the ADEP1 were 18.60 and 14.38 nm, respectively (**Figure 3.5 C**). On the other hand, the measured structural diameter of the LepClpP1^{S98A}P2 in the presence (15 µM) and absence of the ADEP1 were 20.79 and 16.03 nm, respectively (**Figure 3.5 D**). The DLS of the serine mutant LepClpP heterocomplex bound with the ADEP1 (15 µM) inferred a substantial expansion in the diameter (D_h), where the difference was more striking in the LepClpP1^{S98A}P2 compared to the LepClpP1P2 and the LepClpP1P2^{S97A} variant (**Table 3.1**). The significant change in the structural diameter of the LepClpP and its mutant variants in the presence of the ADEP1 indicates a conformational transformation in the whole LepClpP machinery.

Table 3. 1. Measured hydrodynamic diameter (D_h) of the LepClpP1P2 and its mutant heterocomplex variant in the presence (+) and absence (-) of the ADEP1

LepClpP heterocomplex variants	Hydrodynamic diameter (nm)	
	ADEP1 (-)	ADEP1 (+)
LepClpP1P2	13.60±0.27	16.76±0.22
LepClpP1P2^{S97A}	14.38±0.14	18.60±0.75
LepClpP1^{S98A}P2	16.03±0.95	20.79±0.96

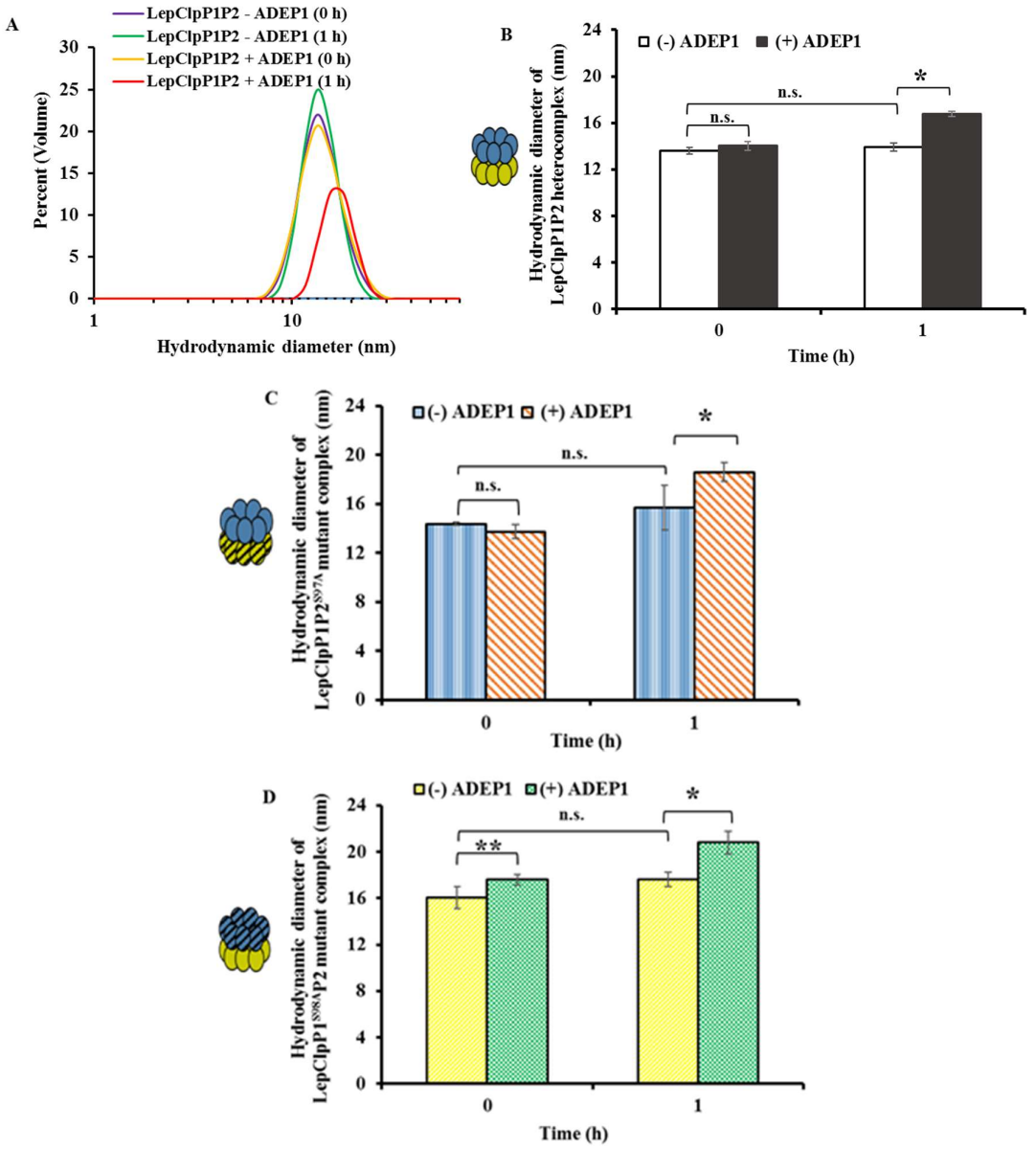


Figure 3. 5 DLS analysis of the LepClpP heterocomplex variants in the presence and absence of the ADEP1.

(A) The representative DLS curves of the LepClpP1P2 (0.5 mg mL^{-1}) supplemented with (+) or without (-) the ADEP1 (15 µM). The relative volume (in %) versus particle size in nanometer (hydrodynamic diameter, D_h) is plotted. The yellow and red curves represent the DLS curves of the LepClpP1P2 in the presence (+) of the ADEP1 treatment at 0 h and 1 h, respectively. The violet and green curves represent the DLS curves of the LepClpP1P2 in the absence (-) of the ADEP1 treatment at 0 h and 1 h, respectively. (B) Comparison of the average hydrodynamic diameter (D_h) of the LepClpP1P2 in the presence and absence of the ADEP1 at different time intervals (0 h and 1 h) using a bar graph. (C) Comparison of the average D_h of the mutant heterocomplex ($LepClpP1P2^{S97A}$) in the presence and absence of the ADEP1 at different time intervals (0 h and 1 h). (D) Comparison of the D_h of the mutant heterocomplex ($LepClpP1^{S98A}P2$) in the presence and absence of the ADEP1 at different time intervals (0 h and 1 h).

The schematics with a diagonal line through them are the LepClpP1^{S98A} (blue with black diagonal lines) and the LepClpP2^{S97A} (yellow with black diagonal lines). The error bars represent the standard errors of the mean (SEM) from the two-independent experiments, where N = 15, the number of technical replicates for each of the protein complex in each of the experiments. Student's t-test performed for statistical analysis to compare the measured D_h values (**p-value<0.005; *p-value<0.05; n.s. as not significant).

3.3.5 Serine^{98}, a catalytic triad residue of the LepClpP1 in the LepClpP heterocomplex, is critical for the ADEP1 mediated activation

It has been hitherto unknown whether the two LepClpP isoforms are indistinctly susceptible to the ADEP1. The measured protease activity of the LepClpP1P2 and its structural hydrodynamic diameter were more in the presence of the ADEP1 than in the absence of the ADEP1. Moreover, the ADEP1 bound LepClpP1P2 is functionally a LepClpX independent caseinolytic protease. This indirectly points towards the relaxed structural state of the LepClpP1P2 in the presence of the ADEP1, which may arise due to the binding towards the apical surface of the LepClpP hydrophobic pocket. Interestingly enough, the DLS of the serine mutant LepClpP heterocomplex bound with the ADEP1 (15 μM) made us witness the increase in the diameter (D_h) to be more articulated in the LepClpP1^{S98A}P2 compared to the LepClpP1P2 and the LepClpP1P2^{S97A} variant. To explore the correlation of the increase in the diameter of the LepClpP active site mutant variants of each isoform with its functional activity in the presence of the ADEP1, we aspired to gauge and compare its activity. Time-dependent casein (fluorescently labeled and unlabelled) degradation assay using the mutant LepClpP heterocomplex (LepClpP1P2^{S97A}) bound to the ADEP1 demonstrated a gain in the protease activity (**Figure 3.6 A** and **3.6 B**) in comparison to the mutant heterocomplex in the absence of the ADEP1. However, the ADEP1 bound LepClpP1^{S98A}P2 failed to illustrate any gain in protease activity (**Figure 3.6 C** and **3.6 D**), and so did the LepClpP1^{S98A}P2^{S97A} (data not shown). Such differential biochemical and the biophysical properties of the reconstituted LepClpP mutant heterocomplex within the active site variants of isoforms imply that the LepClpP displays an additional level of susceptibility towards the ADEP1. The residue serine 98 of the LepClpP1 is the preferred catalytic site of the ADEP1 interaction compared to the ClpP2 of the heterocomplex. These data further reflect that the LepClpP1 active sites are more critical than the LepClpP2's in cleaving the model casein substrate and the active sites of the LepClpP1 are a plausible location for the ADEP1 interaction.

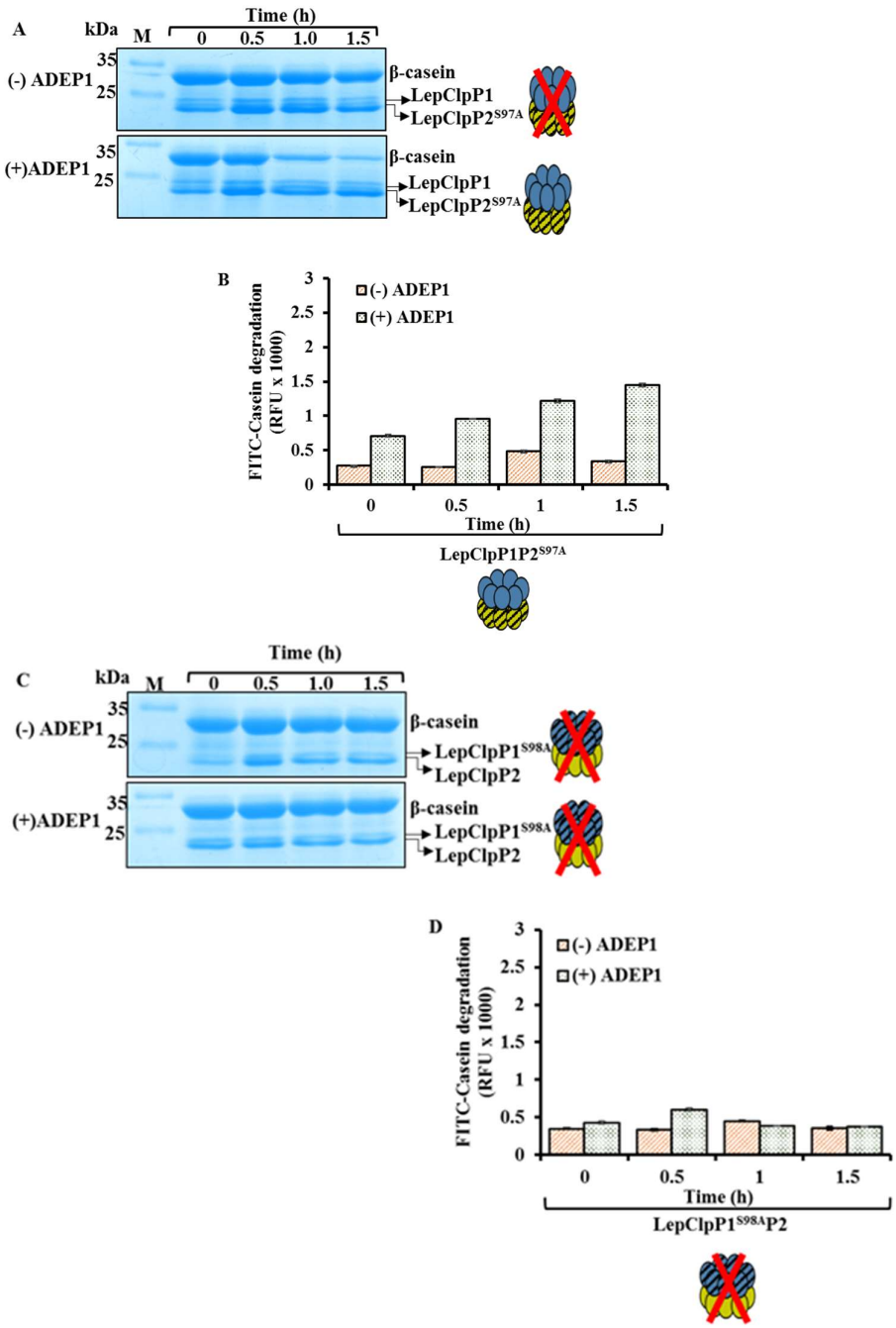
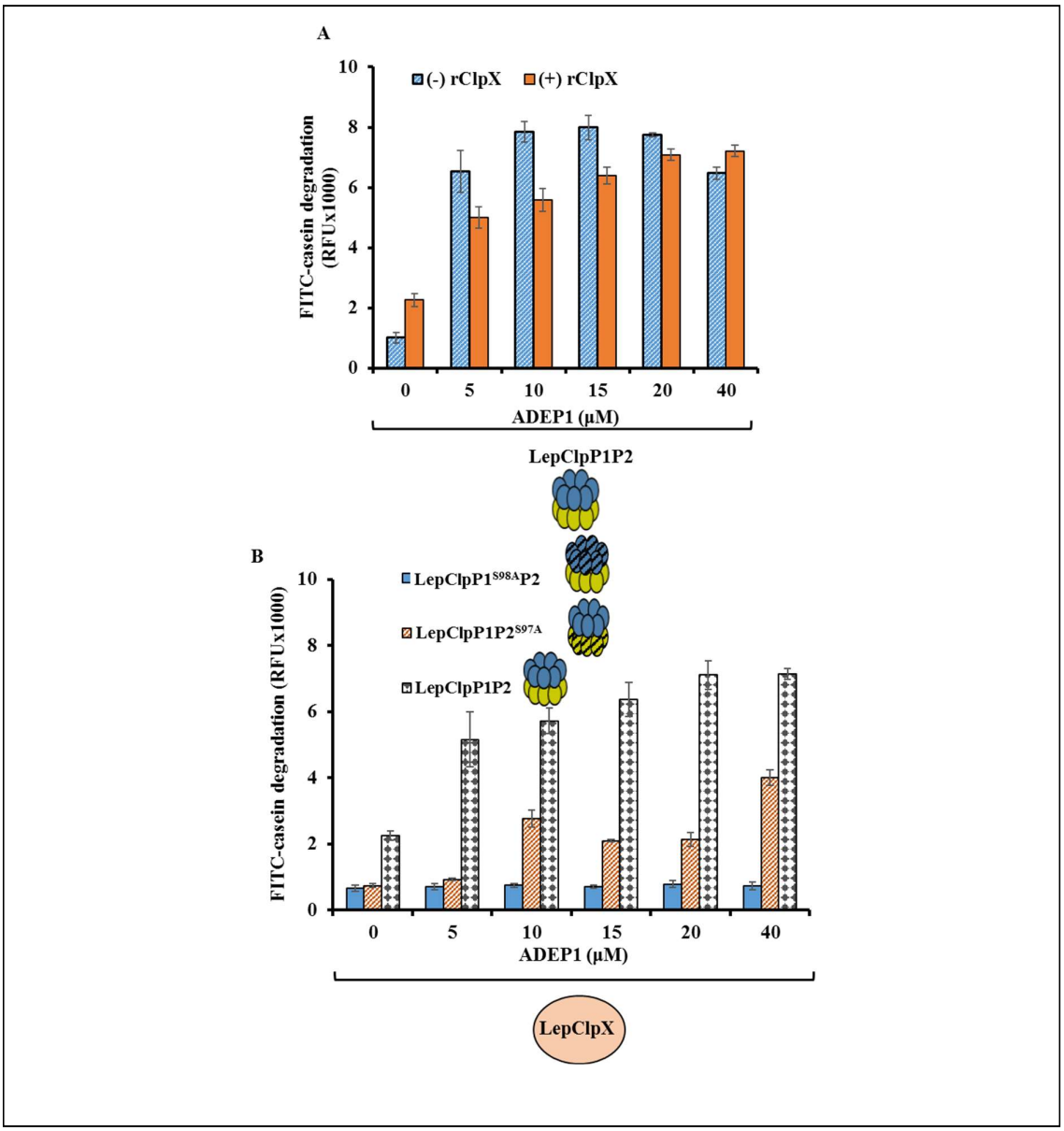


Figure 3. 6. ADEP1 demonstrates predisposed interaction to the LepClpP1 isoform to configurationally switch on the ClpP machinery.
(A and B) ADEP1 stimulates the protease activity of the mutant LepClpP1P2^{S97A} heterocomplex on the β-casein and the fluorogenic FITC-casein substrates, respectively. With the increasing time of the ClpP protease reaction, the ADEP1 stimulates the mutant heterocomplex LepClpP1P2^{S97A} to degrade β-casein (shown on the polyacrylamide gel stained with the Coomassie-blue) and the FITC-casein substrates (shown in fluorescence measurements). **(C and D)** ADEP1 fails to stimulate the mutant LepClpP1^{S98A}P2 heterocomplex's protease activity on the β-casein and the fluorogenic FITC-casein substrates, respectively. The error bars represent the standard errors of the mean (SEM) from the two independent experiments performed.

3.3.6 ADEP1 enhances the LepClpXP1P2 complex activity.

We have illustrated previously that the LepClpXP1P2 complex is a caseinolytic protease in ATP's presence (Dhara et al. 2019). Hence, we assessed the impact of the ADEP1 supplementation on the activity of the LepClpXP1P2 complex. The protease reaction illustrated a refinement in the degradation of FITC-casein by the LepClpXP1P2 complex in the presence of the increasing concentrations of the ADEP1 (5 - 20 μM), and afterward, the protease activity attained saturation (**Figure 3.7 A**). Interestingly enough, without the aid of an ATPase chaperone (LepClpX) and in the presence of the ADEP1 (5 - 20 μM), the LepClpP1P2 showed more (4-fold) enhancement of the activity or can assert rampant stimulation than the LepClpXP1P2 complex (**Figure 3.7 A**). We also noticed a relatively lower protease enhancement of the LepClpP1P2 than the LepClpXP1P2 at the higher ADEP1 concentration (40 μM), implying the fallout of the ADEP1 on the LepClpP1P2 differs at its higher concentration, and the presence of the LepClpX regulates the LepClpP1P2 activity decently in the presence of the ADEP1 (**Figure 3.7 A**). The justification for the straight enhancement of LepClpXP1P2 activity in the presence of ADEP1 may be due to the simple increase in axial diameter of LepClpP1P2, but it seems dubious as LepClpX and ADEP1 compete for the same hydrophobic sites. The second likelihood could be the ADEP1 interaction at the catalytic serine 98 residue of the LepClpP1 (**Figure 3.6 A** and **3.6 B**), and the third possibility could be that ADEP1 interacts with other unconventional sites in the LepClpP1P2. We formulated an independent protease assay to address the first two possibilities. We used the serine mutants LepClpXP1P2 complex (LepClpXP1^{S98A}P2 and LepClpXP1P2^{S97A}) in the presence of an increasing concentration of the ADEP1 (5 - 40 μM) and compared with the protease activity of the LepClpXP1P2 (**Figure 3.7 B**). In consensus with our protease reaction embodied in **Figure 6**, a reclaim of the protease activity could be detected exclusively in the mutant LepClpXP1P2^{S97A} complex in the presence of the ADEP1, though it was lower than the LepClpXP1P2 complex (**Figure 3.7 B**). The protease reaction advocates that the regain in mutant LepClpXP1P2^{S97A} complex's protease activity is predominantly due to the interaction of the ADEP1 at the catalytic serine 98 residue of LepClpP1 and not to that of LepClpP2 of the heterocomplex. A modest expansion in the axial pore diameter of the LepClpP1P2 tetradecamer (by LepClpX) could not activate the machinery. To further corroborate the analysis that the increase in the machinery's axial pore diameter is not the solitary cause for an increase in the protease activity, an independent time chase protease experiment was executed. The time chase (0-1.5 h) protease assay using the mutant LepClpP1P2^{S97A} in the presence of the ADEP1 (15 μM) was compared with the LepClpXP1P2^{S97A} complex (**Figure 3.7C**). There

was no statistically meaningful difference Figured in the protease activity of both the LepClpP1P2^{S97A} heterocomplex and the LepClpXP1P2^{S97A} complex in the presence of the ADEP1 (**Figure 3.7 C**). Collectively, the LepClpP1P2 activity enhancement comes off to be because of the ADEP1 interaction to the active residue serine 98 of the LepClpP1.



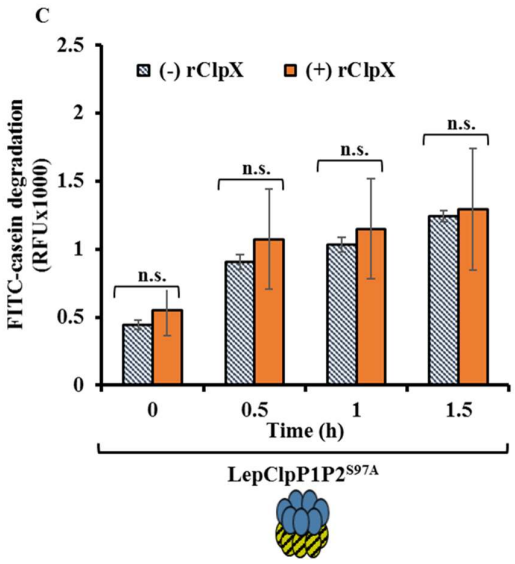


Figure 3. 7. ADEP1 enhances the protease activity of the LepClpXP1P2 and demonstrates biased catalytic activation of the mutant LepClpXP1P2^{S97A} complex.
(A) The substrate FITC-casein degradation (using fluorescence measurements) by the LepClpP1P2 and the LepClpXP1P2 complex in the presence of ADEP1. Increasing concentration of the ADEP1 (0 - 40 µM) enhanced the degradation of FITC-casein by the LepClpXP1P2 complex. **(B)** Comparison of LepClpXP1P2 complex the protease activity with that of the serine mutant's complex (LepClpXP1^{S98A}P2 and LepClpXP1P2^{S97A}) in the presence of ADEP1. The measured fluorescence in the absence of ADEP1 for the LepClp1P2 reflects the default background reading. **(C)** Comparison of a time chase (0-1.5 h) protease assay between the mutant LepClpP1P2^{S97A} and the LepClpXP1P2^{S97A} complex in the presence of the ADEP1. There was no significant difference statistically (p-value >0.05; n.s.) in the rate of protease activity between the mutant LepClpP1P2^{S97A} and LepClpXP1P2^{S97A} complex in the presence of an optimum concentration of the ADEP1 (15 µM). The error bars indicate the respective standard errors of the mean (SEM) from the two independent experiments performed.

3.3.7 Model of the LepClpP1P2 structure reflects the hydrophobic pocket lay in the LepClpP2 subunits

In our earlier study, we showed that the various critical motifs of the LepClpP isoforms crucial for conducting regulation are highly conserved in comparison to its known orthologs like the catalytic triad, Tyr activation trigger, Asp (Glu)/Arg oligomerization sensor domains, and the Gly-rich heptamer dimerization domain (Dhara et al. 2019). In this study, the tertiary structure model of the LepClpP1P2 tetradecamer was developed using computational approaches. Although the ClpP1 and the ClpP2 subunits of the *M. tuberculosis* and the *L. interrogans* share a low sequence identity (~40%), their tertiary structures are very similar. A structural comparison of the ClpP1 and the ClpP2 subunits of the *Mycobacterium* and the *Leptospira* reveals that they are quite analogous with an average root mean square deviation (rmsd) of ~0.6 Å. A representative model of the LepClpP1P2 complex, along with the predicted potential ADEP1 binding hydrophobic pocket, indicates a similarity between the *Mycobacterium* and

the *Leptospira* (**Figure 3.8 A** and **3.8 B**). In the LepClpP1P2 heterotetradecamer model, the hydrophobic sites are exclusively present in the LepClpP2 heptamer apical region (**Figure 3.8 A** and **3.8 B**). It is the hydrophobic binding sites of the LepClpP1P2 where the chaperone LepClpX or the ADEPs may dock to constitute the operative protease. A comparison of the axial pore of the crystal structure of MycClpP1P2 complex with that of the modeled LepClpP1P2 structure shows that its diameter in the former is the same throughout from one to the other end while that in the later is conical from ClpP2 to ClpP1 end. Modeling of the LepClpP1P2 leads us to speculate that identical hydrophobic pockets, as noticed in its orthologs, are prevailing in the LepClpP2 required to bind its physiological chaperone (ATPase) or the antibiotic ADEP.

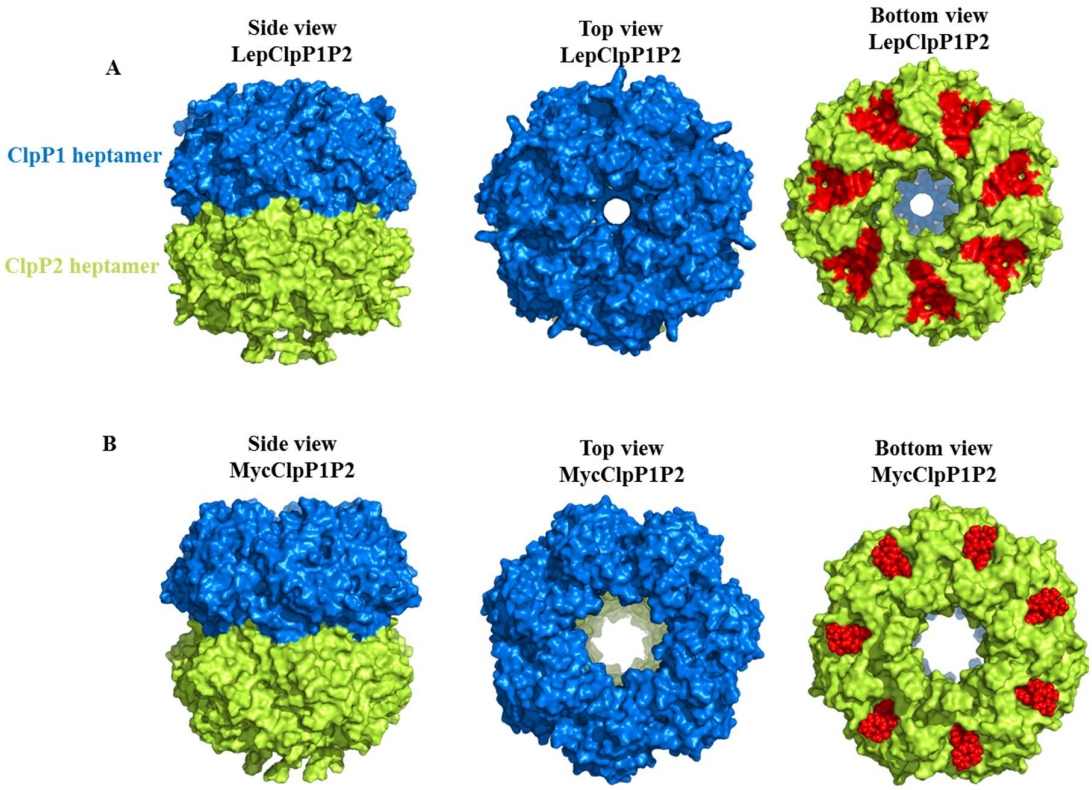


Figure 3. 8. Modeling the tertiary structure of the LepClpP1P2 and its comparison to the *Mycobacterium* ClpP.
(A and B). The surface representation of the model LepClpP1P2 heterocomplex and a crystal structure of the MycClpP1P2 of the *M. tuberculosis*, respectively. The representative side, top, and bottom views of the LepClpP1P2 are compared with the known crystal structure of the MycClpP1P2, respectively. The ClpP1 and ClpP2 heptamer are represented in marine and limon color. The ADEP molecules bound to the MycClpP2 protein is represented as red spheres. A similar hydrophobic site at the LepClpP2 apical region to that of the ADEP binding of the MycClpP2 is highlighted in red, each spanning the two neighboring LepClpP2 subunits at the apical surface.

3.3.8 Ligand ADEP1 show a strong affinity for the ClpP1P2 tetradecamer of *Leptospira*

A thermodynamically favored interaction was obtained when the LepClpP1P2 was titrated into a solution of ADEP1 using isothermal titration calorimetry (ITC). The data analysis yielded an ADEP1: ClpP1P2 molar ratio of approx. 1.0 and a K_d of 0.145 µM or 145 nM (**Fig 3.9**). The data represents a strong binding affinity of an ADEP1 molecule to the LepClpP1P2 tetradecamer of *Leptospira*. The stoichiometric ratio close to value one between ADEP1 and LepClpP1P2 can imply two possibilities; either 14 molecules of ADEP1 bind to 14 subunits of the LepClpP1P2 or seven molecules of ADEP1 bind to 7 subunits of the predicted hydrophobic region of the LepClpP2. In most probability, the interaction of ADEP1 is occurring in two regions of the LepClpP1P2 tetradecamer simultaneously. One interaction at the predicted apical sites of the hydrophobic pocket region of LepClpP2 and other at the active serine residues of the LepClpP1 heptamer, as hinted by the biochemical assays involving serine mutant complex of LepClpP1P2^{S97A}.

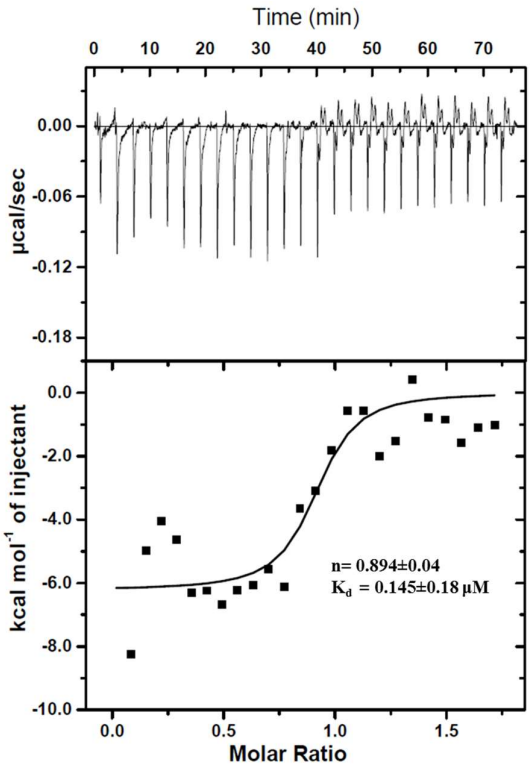


Figure 3. 9. ADEP1 have a strong interaction with LepClpP1P2.
ITC titration of *Leptospiral* ClpP1P2 (200 µM) into a solution of ADEP1 (15 µM). Parameters obtained from this experiment are: stoichiometry factor n= 0.894±0.04 ; Dissociation constant K_d= 0.145±0.18 µM ; Enthalpy ΔH= -6222±434.8 cal mol^{-1} ; Entropy ΔS= 11.2 cal mol^{-1} K^{-1}. The stoichiometry factor 'n' refers to the ratio of monomeric ClpP1P2 and ADEP1. However, the error in the dissociation constant K_d is quite high, thus this result might not be a reliable one.

3.3.9 In LepClpP1P2, ADEP1 docks at a different site than its original apical site of ClpP2 ortholog

Further, to understand the paradoxical effect of ADEP1 on LepClpXP1P2, molecular docking simulation was performed on the model LepClpP1P2 tetradecamer structure composed of two stacked homogenous heptameric rings of ClpP1 and ClpP2 to identify the ligand (ADEP1) binding site and to estimate the binding affinity. Initially, to validate the docking simulation, molecular docking calculations of ADEP (a derivative of ADEP1) on MycClpP (MycClpP1P2, tetradecamer, PDB id: 4u0g) and ADEP1 on ClpP of *Bacillus subtilis* (BacClpP, heptamer, PDB id: 3kti) were carried out. Results confirm the binding of ADEP and ADEP1 at their desired apical site (hydrophobic pocket) as per the ligand-bound ClpP crystal structure available, respectively. The lowest mean binding energy of ADEP to MycClpP1P2 and ADEP1 to BacClpP proteins calculated were -10.54 and -11.26 kcal mol^{-1}, respectively (**Fig 3.10**).

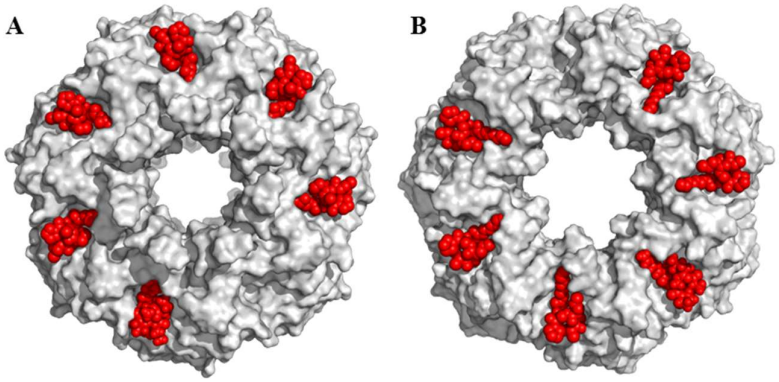


Figure 3. 10. Molecular docking of ADEP and ADEP1 to the ClpP1P2 tetradecamers of *M. tuberculosis* and *B. subtilis* respectively.
Both the MycClpP2 and BacClpP2 proteins are represented in grey color. **(A)** The surface representation of the top-view of *M. tuberculosis* ClpP1P2 (MycClpP1P2) crystal structure bound to ADEP after molecular docking. The lowest mean binding energy of ADEP to MycClpP1P2 calculated was -10.54 kcal mol^{-1}. The interacting amino acids are Lys35, Leu36, Glu39, Leu61, Val62, Ser65, Thr73, Tyr75, Thr92,Tyr95, Arg97, Gln101, Val103, Leu105, Met125, Leu127, Leu204. The ADEP molecules bound to the MycClpP2 protein is represented as red spheres. **(B)** The surface representation of the top-view of *B. subtilis* ClpP (BacClpP) crystal structure bound to ADEP1 after molecular docking. The lowest mean binding energy of ADEP1 to BacClpP calculated was -11.26 kcal mol^{-1}. The interacting amino acids are Arg22, Leu23, Ile28, Val44, Leu48, Phe49, Ala52, Tyr62, Thr79, Phe82, Ile90, Ile92, Leu114, Leu189. The ADEP1 molecules bound to the BacClpP protein is represented as red spheres. In both the cases , the ADEP molecules dock at the desired hydrophobic pockets on the apical sites of the tetradecamer.

Subsequently, the docking calculations of ADEP1 on *Leptospira* ClpP1P2 tetradecamer were executed. Results reveal that ADEP1 binds on another hydrophobic pocket of ClpP2 protein of the heterocomplex lying at the axial face of the ClpP2 heptameric ring spanning two ClpP2 units rather than the conventional hydrophobic pocket at the apical site in other orthologs (**Fig 3.11 A, top and side view**). The calculated lowest mean binding energy of ADEP1 at this new

hydrophobic pocket of *Leptospira* ClpP2 was -8.11 kcal mol^{-1}. Strikingly, none of the top ten-ranked docked conformations of ADEP1 binds at the known hydrophobic pocket (apical site) of the ClpP2 protein constituting the heterocomplex. A representative of the single ADEP1 docked molecule to the new hydrophobic pocket spanning the two ClpP2 subunits of *Leptospira* is shown (**Fig 3.11 B**). Thus, the docking calculations indicate a different ADEP1 binding hydrophobic pocket in the ClpP1P2 heterocomplex of *Leptospira,* and ADEP1 may not compete and block ClpX binding hydrophobic pocket. Although we cannot rely on this MD simulation study, nevertheless, gain in the caseinolytic activity of the LepClpXP1P2 complex in the presence of ADEP1 is in agreement with the MD simulation.

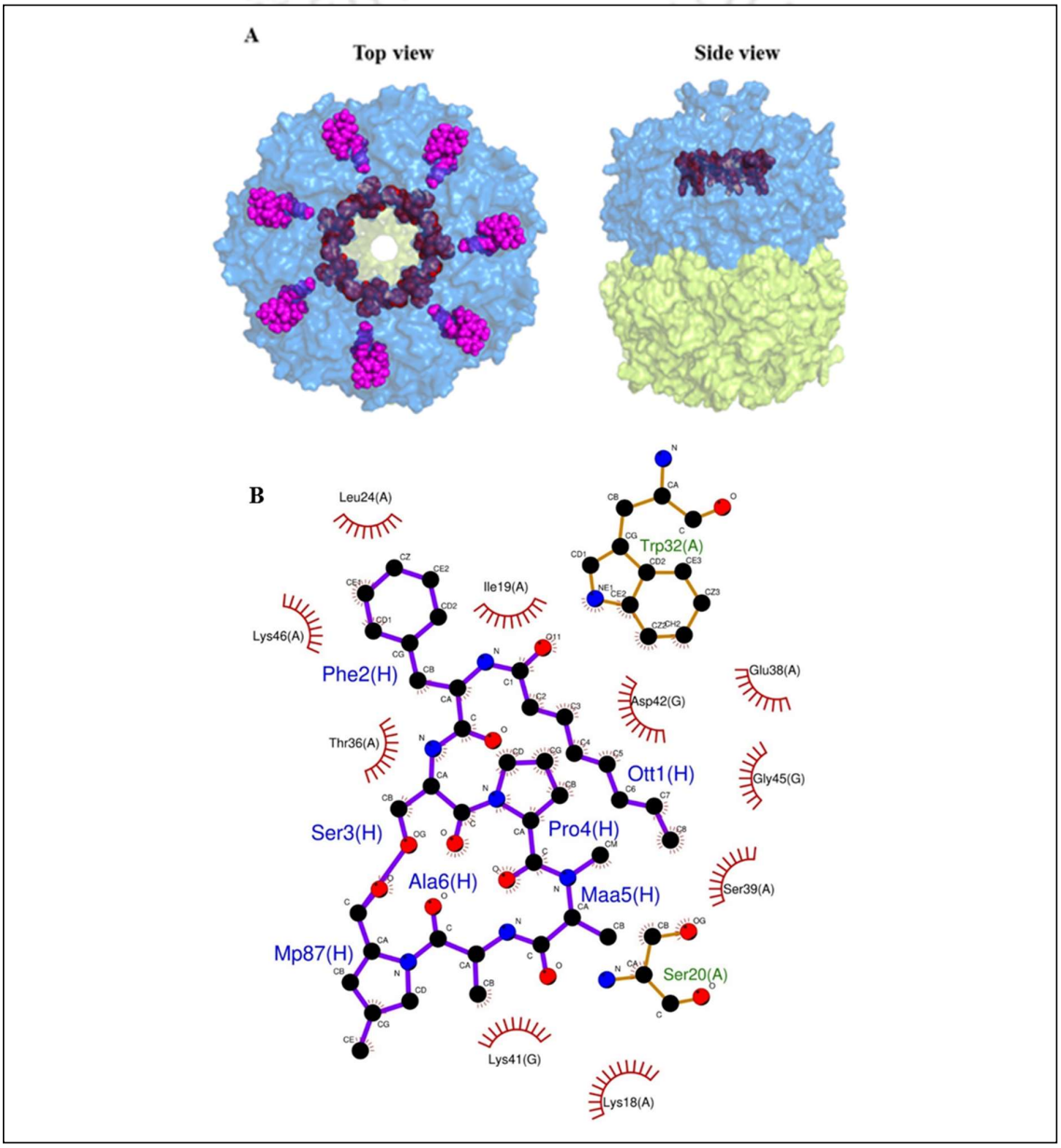


Figure 3. 11. Binding of ADEP1 to the ClpP1P2 heterocomplex of *Leptospira*.
(A) Top and side view representations of the modelled ClpP1P2 structure (tetradecamer) bound to ADEP1 of *Leptospira* after molecular docking. The proteins ClpP1 (heptamer) and ClpP2 (heptamer) modelled structure are shown as surface representation in green and blue, respectively of the tetradecamer. A total of seven ADEP1 molecules docked to the ClpP2 heptameric ring at the identical inner-axial surface with lowest binding energy, each spanning the two neighbouring ClpP2 subunits are shown as spheres in red (appearing reddish-purple due to superimposition). The enforced ADEP1 binding at the apical site is displayed as spheres in magenta spanning the two neighbouring ClpP2 subunits to differentiate the new ligand site with the conventional hydrophobic pocket (outer apical site) of ClpP2 heptamer. **(B)** Interactions of one of single ADEP1 molecule binding with the two neighbouring ClpP2 subunits residues of *Leptospira* with the lowest binding energy. The list of interactions of ADEP1 docking to the ClpP2 proteins is shown here. The amino acid residues having polar (Ser20, Trp32) and non-polar (Lys18, Ile19, Leu24, Thr36, Glu38, Ser39, Lys41, Asp42, Gly45 and Lys46) interactions with the ligand are labelled in green and black, respectively. The ligand ADEP1 is shown as a ball-and-stick model and labelled in blue.

3.4 DISCUSSION

The Clp protease system as a target of antibiotic acyldepsipeptides (ADEPs) can be exploited for efficient bacterial killing either by the opening of the axial pore of the ClpP (Brotz-Oesterhelt et al. 2005, Thomy et al. 2019) or by impeding the ClpP-ATPase chaperone interaction that disrupts regulated proteostasis (Famulla et al. 2016, Fetzer et al. 2017, Weinhäupl et al. 2018). Specifically, activation of ClpP has been validated and proven safe *in vivo* as an antibacterial strategy against systemic lethal infections of *Enterococcus faecium*, *E. faecalis* (vancomycin sensitive and -resistant), *S. aureus* (methicillin resistant and -sensitive), and *S. pneumonia*. In each case, activation of ClpP ADEP outperformed clinically utilized antibiotics, including linezolid and ampicillin (Lavey et al. 2018). For example, the synthesized congeners of the first ADEPs (ADEP2 and ADEP4) were tested in infected mouse models either via intraperitoneal or intravenous injection. The bactericidal activity against *S. aureus*, *E. faecalis*, and *S. pneumoniae* in the infected mice was increased 160-fold compared to ADEP1. The drug's ADME (Absorption, Distribution, Metabolism, and Excretion) properties were also desirable compared to the first ADEPs (ADEP1 and Enopeptin A) with moderate to high clearance and distribution with half-life of 1-2 h in mice and dogs (Brotz-Oesterhelt et al. 2005). ADEP4 inhibited the growth of commensal isolates of *E. feacalis* and *E. faecum* and mice models infected with resistant strains of enterococci showed a significant decrease in disease burden within 24 h of administration of ADEP4 compared to the untreated controls (Brown Gandt et al. 2018). Nonetheless, the level of the susceptibility of the ClpP and its isoform towards the ADEP1 or its derivative are inconsistent as per the genus of the bacteria it belongs (Lavey et al. 2018, Schmitz et al. 2014, Pan et al. 2019). In the current study, as proof of the concept, we chose to use natural antibiotic ADEP1 to investigate the molecular

functioning of the spirochetes' ClpP protease. The minimal inhibitory concentration (MIC) of the ADEPs for testing the *Mycobacterial* growth is in the range of 16 - 64 μg mL^{-1} (~23-90 μM), whereas in the *B. subtilis,* it is at a substantial lower range (nanomolar) (Famulla et al. 2016). In this investigation, the *Leptospira* growth was compromised in the presence of 43 μg mL^{-1}ADEP1 (60 μM) along with lengthening in its morphology. Of note, in the *L. interrogans,* there is another closer ATPase dependent Clp protease (a threonine protease, LepClpYQ, or HslUV), deletion of which ushered a failure of its survival in the hosts and the transmission of the leptospirosis (Dong et al. 2017). Consequently, the application of the ADEP1 for controlling the leptospirosis by targeting LepClpP as an alternative to traditional drugs may be persuading.

The documented impact of ADEP on the multiple ClpP isoforms has been inconsistent under *in vitro* conditions. For instance, a synthetic ADEP could stimulate the peptidase activity specifically to only one pure CloClpP isoform (ClpP1) of the *C. difficile* (Lavey et al. 2018). On the other hand, in this study, ADEP1 did not turn on any of the pure LepClpP isoforms even though the pure isoforms can oligomerize in the absence of the ADEP1 (Dhara et al. 2019). The natural ADEP1 or its synthetic derivative has been established to activate the peptidase activity in both the single and multiple variants of the ClpP orthologs (Brotz-Oesterhelt et al. 2005, Famulla et al. 2016, Gersch et al. 2015). The relative inhibition in the peptidase activity of the operative LepClpP1P2 (short-preincubated) in the presence of the ADEP1 was thus unanticipated in this study.

On the contrary, LepClpP1P2 (long-preincubated), despite encountering self-cleavage, LepClpP subunits exhibited a relative gain in peptidase activity in the presence of ADEP1 than the basal activity. With the numerous shreds of information obtained in this investigation and from the previous analysis (Dhara et al. 2019), the relative decline in peptidase activity of LepClpPs (short-preincubated) in the presence of ADEP1 could be due to many factors like the self-cleavage of the LepClpP protomers or the availability of a minor population of the stable/operative LepClpPs. It is striking that on supplementation of the casein or BSA protein to the LepClpP1P2 (short-preincubated), the peptidase reaction in the presence of ADEP1 detected reversal in the inhibitions (2.6-3.4 fold activation). We believe the LepClpP peptidase activity is directly proportional to the number of stable and operative self-assembled LepClpP machinery. This is substantiated in an identical LepClpP protease/peptidase experiment (**Figure 3.4 C**, **Appendices B.2 B**, and **B.3 C**) wherein the supplementation of casein or BSA resulted in abolition in the self-cleavage of the LepClpP protomers by the ADEP1 activated operative LepClpP1P2 (short-preincubated).

During the short-preincubation period of the LepClpP isoforms, the dynamic equilibrium state between the operating heterotetradecamer LepClpP machinery and its free LepClpP protomers, there is a small number of the operating stable LepClpP peptidase machinery (Dhara et al. 2019). The ADEP1 is known to bind to the hydrophobic pocket on the outer edge of the apical surface of the ClpP, and once engaged at the interface between the adjacent monomers, it yields an incitation and the widening of the apical pore of the ClpP protease (Lee et al. 2010, Gersch et al. 2015, Kirstein et al. 2009). In the LepClpP, the operative LepClpP machinery (short-preincubated) gets stimulated by the ADEP1, and due to an increase in the diameter of the apical substrate channel, degradation of its free LepClpP (more abundant) protomers may transpire. As the short-preincubated LepClpPs are unstable, the LepClpP subunits degradation may shift the dynamic equilibrium towards the free subunits leaving only a few operative LepClpP1P2 (short-incubated) peptidase machinery. Our assumption agrees with an earlier investigation (Dhara et al. 2019), where the binding affinity between the two LepClpP isoforms was moderate in range (K_d=2.02 ± 0.1 μM). Numerous other time-dependent structure stabilization of the ClpP orthologs have been debated elsewhere (Lavey et al. 2018, Famulla et al. 2016). The CloClpP2 assembled into a tetradecameric complex at ≥48 h of incubation (Lavey et al. 2018) whereas, an incubation time of 4 h was a prerequisite in displaying catalytic activity in the MycClpP1P2 (Famulla et al. 2016).

The influence of the ADEP1 is not equally binding to operative LepClpP1P2 (long-preincubated) wherein a surplus number of the stable and operative tetradecamer population is recorded (Dhara et al. 2019). In the same study, the peptidase activity of the LepClpP1P2 (long-preincubated) was higher than the LepClpP1P2 (short-preincubated) at a given time point (Dhara et al. 2019). In concordance, the LepClpP1P2 (long-preincubated) peptidase activity was more enhanced with the ADEP1. The stimulation fallout of the ADEPs on the ClpP also depends on other factors like the form of the ADEP used as an activator, catalytic acceleration at a ClpP serine residue, and the structural stability of the self-assembled ClpP (Lee et al. 2010, Gersch et al. 2015, Lavey et al. 2018, Schmitz et al. 2020). In consensus to this, despite ADEP1 mediated self-degradation of LepClpP protomers in the LepClpP1P2 (long-preincubated), there was a relative gain in the peptidase activity than the basal activity. The ADEP1 mediated LepClpP1P2 (long-incubated) peptidase activity enhancement is in concordance to the earlier analyses on the ClpP orthologs elsewhere (Brotz-Oesterhelt et al. 2005). This investigation hence furnishes directives that another ADEP dependent catalytic activation site in the stable LepClpP1P2 tetradecamer (long-preincubated) might supersede the influence of self-degradation. The catalytic activation of the StaClpP by the ADEP1 is documented in *S. aureus*

with the aid of chemical probes where the ADEP1 stimulates the ClpP activity through the cooperative binding (Gersch et al. 2015). In the same study, it is suggested that ADEP1, besides opening the axial pore of SauClpP by occupying the hydrophobic pocket, also brings about conformational changes into a more active SauClpP. The DLS exploration of the LepClpPs in the presence of the ADEP1 ascertained the conformational changes into a relaxed and active state. The addition of the ADEP analog to MycClpPs (ClpP1P2^{S110}), possessing a mutation in the MycClpP2 active site serine (Ser110), resulted in the salvage of its protease activity but not the MycClpPs (MycClpP1^{S98}P2) variant (Schmitz et al. 2014). Likewise, in this study, it is apparent that the catalytic activation by the ADEP1 happens to be prejudiced towards the LepClpP1 as a mutation in the serine 98 residue stemmed in the complete abolition of the protease activity. At the same time, ADEP1 binding to the LepClpP1P2^{S97A} displayed retention of the activity. While the ADEP1 mediated ClpP1 activation of catalytic serine seems to be equally crucial to the *Mycobacterium* and the *Leptospira* ClpPs, there is a conditional extra peptide agonist required for the MycClpPs activation (Schmitz et al. 2014). The ADEP catalytic activation or a gated-pore process activation of the ClpPs has been noted even in multimeric compartmentalized proteases (Sousa et al. 2000, Groll et al. 2000, Sousa et al. 2002). Lately, a proteasome inhibitor bortezomib has also been recorded to bind to the TheClpP active-sites serine site (*Thermus thermophiles*), emulating a peptide substrate and, evokes activity in the complex (Felix et al. 2019). Another accepted ClpP peptidase inhibitor, β-lactones, bind specifically to the StaClpP catalytic triad to abolish the activity of the StaClpP; however, it fails to abolish in the presence of the ADEP analog (ADEP7) (Gersch et al. 2015).

It is suggested that a total of seven to fourteen ADEP molecule binds to the ClpP complex strictly where the ATPase chaperone engages with the functional ClpP tetradecamer (Wood et al. 2019, Li et al. 2016, Alexopoulos et al. 2013). The number of ADEP1 molecules binding to the ClpP machinery relies on the availability of the hydrophobic pockets and whether the ClpP tetradecamer machinery is composed of two similar heptamers (ClpP1P1$_{7+7}$) or with the two different homogenous heptamers (ClpP1P2$_{7+7}$) stacked one above the other (Li et al. 2016, Wood et al. 2019, Gersch et al. 2015). The hydrophobic pocket thus signifies a hot spot for the ClpP modulation of the various organisms viz. *B. subtilis* (Li et al. 2010), *C. difficile* (Lavey et al. 2018), and *E. coli* (Alexopoulos et al. 2012, Sowole et al. 2013). Interestingly, *Mycobacterium* and *Chlamydia* that encode two ClpP isoforms, the competent ADEP analogs bind to the seven hydrophobic pockets of the ClpP2 heptamer rather than the ClpP1 of the functional tetradecamer (Li et al. 2016, Wood et al. 2019, Schmitz et al. 2020). The MycClpP1P2 binding site for ADEP is correlated with the modeled LepClpP1P2 in this study.

The computationally derived tertiary structure of the LepClpP1P2 infers that the ADEP1 can bind only to the hydrophobic pockets (7 in number) existing in the LepClpP2 heptamer but not the LepClpP1 heptamer. The biased ADEP binding towards the MycClpP2 hydrophobic pocket of the operative MycClpP heterocomplex also steers in simultaneous pore opening in the opposite MycClpP1 ring implying structural interdependency (Schmitz et al. 2014, Gatsogiannis et al. 2019). The axial diameter of the ADEP-bound MycClpP1P2 with peptide agonist is indistinguishable from one end to the other while the modeled LepClpP1P2 structure shows the axial diameter of the complex from the LepClpP2 to LepClpP1 end to be conical. Besides, the primary sequence alignment of the LepClpP2 with the MycClpP2 reveals to be shorter at its N-terminal end by eight residues, whereas the ClpP1 is of proportionate size (Dhara et al. 2019). Admittedly, it is too early to count on a model structure of the LepClpP1P2 unless the crystal structure of the LepClpP1P2 bound to ADEP1 is developed.

In the genus *Pseudomonas* spp, the PseClpP1 functionally interacts with the PseClpX, whereas the PseClpP2 exhibits no evidence of interaction with PseClpX (Hall et al. 2017), portraying the existence of a variant pattern of the Clp orthologs in nature. The measured gain in the LepClpXP1P2 complex activity in the presence of the ADEP1 is in consensus to the EcoClpAP complex activity (Kirstein et al. 2009). Regardless, in the MycClpP, the ADEP can competitively bind to the hydrophobic pocket of the MycClpP2 heptamer and blocks of MycClpX or MycClpC1 engagement with the MycClpP machinery leading to the abolition in the degradation of the natively folded protein GFP-ssrA or the unstructured casein substrate (Schmitz et al. 2014, Famulla et al. 2016). The ADEPs kill many bacterial species by dysregulating the activity of the ClpP such that the multiple cellular proteins are indiscriminately degraded (Kirstein et al. 2009, Lee et al. 2010, Li et al. 2010). In contrast, for *Mycobacterium*, ADEP plays a detrimental role by inhibiting the essential MycClpP-catalyzed proteolysis (Schmitz et al. 2014, Famulla et al. 2016). While drafting this paper, a surprising finding caught our attention wherein a fragment of the ADEPs was deleterious by stimulating the MycClpXP1P2-catalyzed degradation of the cytoplasmic proteins (Schmitz et al. 2020). A reasonable second interaction site at the MycClpP1 catalytic triad for the ADEPs fragment has been proposed in the same study. The role of the second interaction site for ADEP in MycClpPs was in agreement with our finding, although the stimulation of LepClpXP1P2 occurs by intact ADEP1 and without any additional peptide agonists. The lethality of the ADEP1 antibiotics on *Leptospira* growth can thus be assumed to be due to enhanced chaperone-dependent LepClpP protease functions.

The relative higher caseinolytic activity of the LepClpP1P2 in the presence of the ADEP1 than the LepClpXP1P2 complex leads us to speculate that LepClpX may not be dethroned entirely from the hydrophobic pocket by the ADEP1. This can be reasonably illustrated by the disparity in the mode of action of the ADEP1 and the ClpX. Despite being a competitor for the same apical hydrophobic pocket (Lee et al. 2010), ClpX is an ATPase dependent chaperone that would require time to unfold the substrate casein, while ADEP1 works directly on the open-gate model. In one of the ClpXP proteolysis assay investigated elsewhere (Lee et al. 2010), approximately a 2-fold molar excess of the ADEP (calculated in relation to ClpP as a monomer) completely blocks the interaction of the ClpX with the ClpP of both *E. coli* and *B. subtilis*. In contrast to the MycClpXP, a consistent increase in the LepClpXP activity was noted in the presence of the increasing amount of ADEP1, suggesting advancement in indiscriminate degradation of the essential cellular protein. The initiation of auto cleavage of the LepClpP subunits can be a reasonable justification for a relative decline in the protease activity of the LepClpP1P2 at a higher concentration (40 µM) of the ADEP1 than the LepClpPXP1P2. In the presence of the LepClpX, the ADEP1 mediated LepClpP1P2 stimulation comes out to be more controlled. The precise explanation for the relative progress in the protease activity of the LepClpXP1P2 in the presence of the ADEP1 is challenging to comprehend experimentally without any crystal structure. However, it can be conceived that the ADEP1 mediated gain in the protease activity may be due to the additional catalytic activation of the serine 98 of the LepClpP1. We ascertained that LepClpXP1P2^{S97A} in the presence of the ADEP1 could be activated but not LepClpXP1^{S98A}P2. The expansion in hydrodynamic diameter was more pronounced for LepClpP1^{S98A}P2 than LepClpP1P2^{S97A} in the presence of ADEP1; nevertheless, mere gated-pore activation of the LepClpP1^{S98A}P2 did not transpire in any gain of the protease activity.

Additionally, the molecular docking of ADEP1 on modeled ClpP1P2 structure of *Leptospira* leads us to speculate preferential binding of seven ADEP1 molecules at another identical hydrophobic binding pockets of the ClpP2 heptamer, each spanning the two neighboring ClpP2 subunits at the apical axial surface. Moreover, the usual hot spot at the apical site (hydrophobic pocket) of ClpP2 for the ATPase chaperone remains free in the functional ClpP heterocomplex bound to ADEP1. Such MD simulation is in agreement with our observation, where there is an increase in the caseinolytic activity of LepClpXP1P2 in the presence of ADEP1. Admittedly, it is too early to count on MD simulation results built upon a model structure of ClpP1P2 of *Leptospira,* which is not in agreement with the established knowledge of ADEP binding sites accumulated by many other laboratories around the globe. To substantiate the computer

modeling, data evaluation of the ClpP-ADEP co-crystal structure of *Leptospira* is further warranted.

3.5 CONCLUSION

Growth inhibition, biochemical assays, MD simulations, and site-directed mutational analysis of ClpP1P2 bound ADEP1 under *in vitro* condition gave a clue that ClpP can be a suitable Achilles' heel for *Leptospira* by deregulating the proteolysis inside bacteria. The shreds of evidence presented in this study demonstrate that antibiotic ADEP1 can modulate the ClpP system, possibly in an unconventional approach. *Leptospira* ClpP-ADEP co-crystal structure would provide compelling evidence for developing a therapeutic strategy against the spirochetes in the future. Mutation of novel ADEP1 binding site on ClpP2 of *Leptospira* would further implicate the computer modeling in this study. A carefully designed study using various analogs of ADEP1 may help us reduce the MIC of ADEP required for killing leptospires. To our understanding, this is the first study of the ADEP1 mode of action on any pathogenic spirochete in an alternative to a conventional antibiotic. Using ADEP1 as a tool, this study provides an insight into the molecular function ClpP1P2 of *Leptospira* in association with its ATPase chaperone ClpX. While several drug development programs by multiple groups targeting ClpP are underway, no such compounds have yet reached the clinic. Therefore, there is still much work to be done to achieve this goal.

3.6 MATERIALS AND METHODS

3.6.1 Morphology changes and growth assays of *Leptospira interrogans*

L. interrogans were grown *in vitro* at 29°C in 10 mL of the Ellinghausen-McCullough-Johnson (EMJH) medium supplemented with 5-fluorouracil till exponential phase. From the growing culture, 3×10^8 cells were inoculated into the fresh EMJH media (1 mL) with or without the ADEP1 (10 μg mL^{-1} or 15 μM) dissolved in dimethyl sulfoxide (DMSO). The cells' morphology was also investigated by assessing the length of the untreated and treated cells every 24 h till 120 h by the microscopy and imaging software (Zeiss). In an independent experiment the length of 30 treated and untreated *L. interrogans* cells were individually measured to evaluate the change in morphology. For generating the growth curve of the *L. interrogans* in the presence of different concentrations of ADEP1, an exponentially growing culture (100 μL containing 2×10^5 cells) was added to a sterile non-binding white micro-test plate (96-well flat-bottom) in triplicate. Thereupon, to the cultures, ADEP1 was supplemented in the increasing concentrations (0, 20, 40, and 60 μM) and was incubated for 120 h at 29-30°C. The growth of the cells was monitored by counting the cell numbers on a hemocytometer

counting chamber every 24 h till 120 h under the dark field microscopy (20× magnification). Each experiment was executed independently at least twice in triplicate.

3.6.2 Field emission scanning electron microscopy (FESEM) of *L. interrogans*

A 3 mL (6×10^7 cells mL^{-1}) of the exponentially grown culture of the *L. interrogans* in the EMJH medium with or without the addition of 43 µg mL^{-1} of ADEP1 (60 µM) was incubated till 48 h at 29°C. Post incubation, cultures were processed for the FESEM, as illustrated before (Rudenko et al. 2016) with a few modifications. Briefly, the spirochetes were pelleted at 1500× g for 20 min, washed with phosphate buffer saline (pH 7.4), and fixed in glutaraldehyde (5% in 0.1 M phosphate buffer, pH 7.4) for 30 min at room temperature. Fixed samples were rinsed thrice with a phosphate buffer and dehydrated through a graded series of ethanol (35, 50, 75, 95, and 100% for 10 min each), followed by the final drying using hexamethyldisilazane (HMDS, 100%; Sigma) twice with a 10 min of incubation. Each time, cells were recovered by centrifugation. Over-night desiccated specimens (ADEP1 treated and untreated) were individually mounted on the aluminum stubs using double-sided carbon-coated tape, sputter-coated with the gold, and examined under the FESEM (Sigma-300, Zeiss, Germany) operated at 5 kV. The average length of a segment of the three complete spiral turns of ten spirochetes was measured to assess and correlate the partial length of treated and untreated *L. interrogans* in the representative of micrographs.

3.6.3 Overexpression and purification of LepClpP isoforms and LepClpX of *Leptospira*

Caseinolytic protease **(**LepClpP) isoforms and the chaperone LepClpX of the *L. interrogans* serovar Copenhageni were cloned individually in the pET23a, overexpressed and purified from the *E. coli* BL21 (DE3) cells as illustrated before in our laboratory (Dhara et al. 2019).

3.6.4 Peptidase assays of *Leptospira* ClpP isoforms

The LepClpP isoforms mixture (1.5-2 µg) were pre-incubated either for the 10 min at 37°C (short-preincubation) or for the 24 h at 4°C (long-preincubation) in a ClpP peptidase activity buffer (50 mM phosphate buffer pH 7.6, 100 mM KCl, 5% glycerol) to self-assemble into a functional heterocomplex. ADEP1 (BioAustralis, Cat No. BIA-A1570) was dissolved in DEPC-treated water with the 10% DMSO at a given working concentration (100 µM). ADEP1 was added at an increasing concentration (0-40 µM) into the flat bottom black polystyrene 96-well plates (Invitrogen) containing the LepClpP heterocomplex and were incubated for 10 min at 37°C. Fluorogenic dipeptide substrate N-succinyl-Leu-Tyr-AMC (S1: Suc-LY-AMC; Sigma) was added (8 µL of 1 mM) to each of the wells to achieve a final substrate (S1) concentration (100 µM) in a given total reaction volume (80 µL). Assay plates were incubated

for 2 h at 37°C, and the hydrolysis of the fluorogenic dipeptide was monitored via an i-TECAN Infinite M200 plate reader (excitation: 380 nm; emission: 460 nm). When using substrate β-casein in the peptidase activity assay of the short-preincubated LepClpP1P2, the same procedure was followed, as described above, with 28 μM of β-casein (Sigma) in the designated wells. Each experiment was performed at least twice in triplicates. When using proteins β-casein or bovine serum albumin (BSA) in the peptidase activity assay of short- or long-preincubated LepClpP1P2, the same procedure was followed, as described above, with 28 μM of β-casein (Sigma) or BSA (SRL) in the designated wells. Each experiment was performed at least twice in triplicates. The reaction products of long-preincubated LepClpP1P2 peptidase assay in the presence of 15 μM ADEP1 and supplemented with β-casein/BSA were withdrawn at various time-intervals (0- 2 h). The reactions were terminated by adding sample buffer and heating it for 10 min at 95°C. The reaction products at each time point were resolved on 12% SDS-PAGE and visualized by Coomassie staining.

3.6.5 Autoproteolysis assays of LepClpP isoforms

Pure LepClpP isoforms (1.5-2 μg) or its mixture (short- or long-preincubated LepClpP1P2) into the LepClpP peptidase activity buffer were incubated with a varying ADEP1 concentration (0-40 μM) in a given total reaction volume (20 μL) for 2 h at 37°C. Reactions were terminated by adding the sample buffer (SDS-PAGE loading buffer) and heating for 10 min at 95°C. The reaction products were resolved on the 12% SDS-PAGE and visualized by Coomassie staining.

3.6.6 Protease assays of LepClpP isoforms

Pure LepClpP isoforms or their mixture (2 μg) containing short- or long-preincubated LepClpP1P2 in a LepClpP protease activity buffer (50 mM Tris-Cl pH 7.0, 50 mM KCl, 1 mM DTT, 8 mM $MgCl_2$, 5% glycerol) was incubated with the ADEP1 (15 μM) for 10 min at 37°C. After the pre-incubation period, bovine β-casein or BSA (20 μM), wherever applicable, was added to the reaction tube to a given final reaction volume (100 μL). From the total reaction volume, a given small volume (20 μL) of the reaction was terminated at the various intervals (0-1.5 h) after the addition of the sample buffer and heating for 10 min at 95°C. A control reaction of the pure LepClpP isoforms, or its mixture containing an equivalent amount of DMSO to the working solution of ADEP1, was included for comparison. At each time point, the reaction products were resolved on 12% SDS-PAGE and visualized by Coomassie staining. A similar procedure was followed for the β-casein proteolysis assays wherein mutant isoforms of LepClpP (LepClpP1^{S98A} and LepClpP2^{S97A}) were used. In an alternative assay format, LepClpP1P2 and its mutant isoforms were evaluated for the protease activity in the presence

of ADEP1 using fluorogenic substrate FITC-casein (Sigma). The LepClpP1P2 or its mutant mixture (short-preincubated) into the ClpP activity buffer was incubated with the ADEP1 and was added to a 96-well black plate (Invitrogen). To each well, FITC-casein (10 µM) was added to a given final well volume (100 µL). The assay plates were then incubated in the dark for 2 h at 37°C, and the reactions were terminated with the trichloroacetic acid (0.6 N). Hydrolysis of the fluorogenic substrate was monitored via i-TECAN Infinite M200 plate reader (excitation: 492 nm; emission: 519 nm). Readings were obtained at every 0.5 h for 1.5 h. The protease activity of the LepClpXP1P2 in the presence of the ADEP1 was measured using FITC-casein (10 µM) as the substrate in a given (100 µL) total reaction volume. In each of the reaction tube, the short-preincubated LepClpP1P2 (1 µg) were mixed with the LepClpX (2 µg) and incubated with a different ADEP1 concentration (0-40 µM) for 10 min at 37°C. After the incubation period, 4 mM ATP was added to each reaction tube to initiate the protease assay. The downstream of the assay was performed as described for the FITC-casein substrate degradation. A similar FITC-casein degradation assay was carried out as described above to compare the ADEP-bound LepClpXP1P2 and the mutant LepClpXP1^{S98A}P2 or LepClpXP1P2^{S97A} protease activities. A time-chase degradation of FITC-casein measured the activation of the LepClpP1P2^{S97A} tetradecamer and the LepClpXP1P2^{S97A} complex by the ADEP1. The mutant LepClpP1P2^{S97A} (1 µg) and the LepClpX (2 µg) were incubated shortly for 10 min at 37°C and mixed. ADEP1 (15 µM) was taken in a separate tube and further incubated with LepClpP1P2^{S97A} or LepClpXP1P2^{S97A} for another 10 min at 37°C. The reactions were initiated by the addition of FITC-casein (10 µM) and the ATP (4 mM) in a total reaction volume of 100 µL. From the total reaction volume, a given small volume (20 µL) of the reaction was terminated at various intervals (0-1.5 h) with the trichloroacetic acid (0.6 N). Hydrolysis of the fluorogenic substrate was monitored via i-TECAN Infinite M200 plate reader (excitation: 492 nm; emission: 519 nm). Each experiment was performed at least twice in triplicates.

3.6.7 Dynamic light scattering

DLS experiments were performed on a Zetasizer Nano ZS (Malvern Instruments) at 25°C. LepClpP1P2 or its mutant heterocomplex (LepClpP1^{S98A}P2 or LepClpP1P2^{S97A}) (0.5 mg mL^{-1}) were incubated into a buffer (50 mM Tris-Cl pH 8.0, 100 mM NaCl and 10 % glycerol) for 48 h at 4°C for the self-assembly. The LepClpP1P2 with or without ADEP1 (15 µM) was added to the polystyrene cuvettes to record the light scattering. The light scattering was recorded at 173° angle with a 633 nm He–Ne laser as the light source. The LepClpP1P2 and the mutant heterocomplex were restored and further incubated for one h at 37°C, followed by the DLS of

those samples. A total of 15 autocorrelation functions viz. technical replicates were recorded for each protein sample, and the hydrodynamic diameters were determined as described previously (Dhara et al. 2019). Each investigation was performed in duplicate (2× for each of the heterocomplex samples), and the hydrodynamic diameter was measured as the average of these replicates.

3.6.8 Isothermal Titration Calorimetry (ITC)

All ITC experiments were performed on a MicroCal iTC200 system (GE Healthcare) in ITC buffer containing 50 mM Tris-Cl pH 7.5, 125 mM NaCl, 5% glycerol with 1.5% (v/v) DMSO at 37°C and with a constant stirring at 250 r.p.m. Before the experiments, the protein LepClpP1P2 (200 µM) was buffer exchanged into 50 mM Tris-Cl pH 7.5, 125 mM NaCl, 5% glycerol, and concentrated using a 3kDa cut-off centrifugal filter units (Amicon, catalog no. UFC800324). The buffer exchanged LepClpP1P2 was pre-incubated overnight at 4°C to ensure stable tetradecamer formation. The ligand ADEP1 (15 µM) was dissolved in the same buffer from a one mM stock in DMSO. DMSO concentrations of syringe and cell samples were matched by the addition of DMSO in the protein exchange buffer. The sample cell and the syringe were washed twice with ITC buffer before loading the analytes. The equilibrated LepClpP1P2 (60 µL of 200 µM) and the ADEP1 (300 µL of 15 µM) was loaded into the ITC syringe and the sample cell, respectively. The experiment was initiated after equilibration for 300 s with the first injection of LepClpP1P2 (0.4 µL) discarded during the analysis. A typical experiment consisted of 26 subsequent injections with a 1.5 µL injection volume into a cell filled with 300 µL sample. The LepClpP1P2 (1.5 µL) was titrated into the sample cell at an interval of 120 s. Power was recorded at 'high' gain setting with a reference power of 6 µcal s^{-1} and a 5 s filter period. Data analysis, including baseline correction and evaluation, was carried out using OriginPro 8.5 ITC.

3.6.9 Structure prediction of LepClpP1, LepClpP2 and LepClpP1P2

The tertiary structure models of the LepClpP1 and LepClpP2 from *L. interrogans* serovar Copenhageni were predicted using the web-based server Phyre2 (Kelley et al. 2015). Subsequently, the predicted models were refined by the energy-minimization method using the program ModRefiner (Xu and Zhang 2011). LepClpP1, LepClpP2, and LepClpP1P2 oligomers were developed by superposing its monomers onto the known *Mycobacterium tuberculosis* ClpP1P2 structure (MycClpP1P2, PDB id: 4u0g). The stereo-chemical properties of all the refined models were validated using the webserver RAMPAGE (Lovell et al. 2003). All the structural figures were prepared using the program PyMOL.

3.6.10 Molecular docking of ADEP1 to LepClpP1P2

The molecular docking computations were performed using a freely-available program AutoDock version 4.2 (Morris et al. 2009) to estimate the binding affinities of ligand (ADEP1) to the pure ClpP1, ClpP2, and their heterocomplexes. For this, the three-dimensional atomic coordinates of ligands and proteins, wherever available, were extracted from Protein Data Bank (Berman et al. 2000). *Leptospira* ClpP1, ClpP2, and ClpP1P2 oligomers were generated by superposing its monomers onto known ClpP1P2 complex structures from *M. tuberculosis* (MycClpP1P2, PDB id: 4u0g). Hydrogens were added using the module available in the program AutoDock. The partial charges for the protein atoms were assigned using the Gasteiger charge algorithm (Gasteiger and Marsili 1980). The grid size was fixed to 126x126x126 points, keeping a grid-spacing of 1.0 Å, taking the center of mass of the protein as the grid center. In each molecular docking calculation, the protein and ligand atoms were kept rigid, and the blind docking search method was used. For each molecular docking experiment, a default set of parameters were considered, and a total of 2000 runs of the Lamarckian genetic algorithm (GA) was simulated. The docked conformations of ligands were clustered with an RMSD cut-off of 2.0 Å. The list of interactions between the protein and ligand atoms was identified using the web tool PSAP (Balamurugan et al. 2007). The molecular interactions of docked ligands and the protein were visualized using the program LigPlot+ (Laskowski and Swindells 2011), and all other structural figures were prepared using the program PyMOL.

3.6.11 Statistical analyses

All the results are expressed as means ± standard errors of the mean (SEM). Student's paired t-test was used to determine the significance of differences between the means, and the p-values of <0.05 were regarded as statistically significant. At least two independent experiments were performed, each in the duplicate or triplicate, as mentioned in the materials and methods section and the figure legends.

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Chapter 4

Study of ClpP function by mutational analysis

4.1 ABSTRACT

The functional activity of *L. interrogans* caseinolytic protease (ClpP) isoforms mutant variants was analyzed to furnish evidence about the unprecedented activation of ClpPs by acyldepsipeptide. The selected single and double residue mutations of ClpP1 and ClpP2 at conserved hotspots displaying varied functional activities are discussed in this chapter. Out of the five LepClpP mutants (LepClpP1^{N172D}, LepClpP1^{E170D}, LepClpP2^{S40AK41N}, LepClpP2^{Y63A}, LepClpP2^{S40AK41N}), only four (LepClpP1^{N172D}, LepClpP2^{S40AK41N}, LepClpP2^{Y63A}, LepClpP2^{S40AK41N}) got mutated at the desired location. Preliminary studies revealed a gain of function in LepClpP mutants (LepClpP1^{N172D}P2, LepClpP2^{S40AK41N}, LepClpP2^{Y63A}). The mutant LepClpP2 isoforms – LepClpP2^{S40AK41N} and LepClpP2^{Y63A} were functionally active alone without the coupling of pure LepClpP1. But, peptidase activity of the pure LepClpP2^{S40AK41N} variant was ~8-fold lower versus LepClpP1P2. There was gain in the protease activity of pure LepClpP2^{Y63A}, in contrast to the LepClpP1P2. However, pure LepClpP2^{I126G127_del} was neither enzymatically active nor in combination with LepClpP1. The pure LepClpP1^{N172D} mutant was inactive, but it became enzymatically active when coupled with LepClpP2. Nevertheless, the level of peptidase activation was ~7 fold lower versus the LepClpP1P2 heterocomplex.

The genetic manipulation of the Clp system in recalcitrant *L. interrogans* might prove useful in studying the system's biological significance in the spirochete. However, there is difficulty in investigating the causal agent by reverse genetic approach, so we decided to delete one of the *clpP* genes in a saprophytic strain of *Leptospira*. As a result, along with *in vitro* biochemical studies on LepClpP mutants, we also attempted to delete one of the *clpP* genes of *L. biflexa* utilizing double homologous recombination. A suicidal mutagenesis plasmid construct was generated flanking 1000 nt upstream and 1000 nt downstream regions to *clpP2* (*LEPBI_I0969*) gene of *L. biflexa*. We could not succeed in generating an *L. biflexa* Δ*clpP2* mutant despite several attempts.

4.2 INTRODUCTION

To date, our understanding of multiple clpP gene expressing organisms is based primarily upon microbes, *S. elongatus* (Stanne et al. 2007), *L. monocytogenes* (Gaillot et al. 2000, Zeiler et al. 2011, Zeiler et al. 2013, Dahmen et al. 2015, Gatsogiannis et al. 2019), *P. aeruginosa* (Hall et al. 2016) , *M. tuberculosis*,(Akopian et al. 2015, Benaroudj et al. 2011, Famulla et al. 2016, Raju et al. 2012, Schmitz and Sauer 2014, Vahidi et al. 2020, Li et al. 2016), *Clostridium difficile* (Lavey et al. 2018), and *Chlamydia trachomatis* (Pan et al. 2019, Wood et al. 2020) all of which reveal structural disparities and distinct regulation profiles of ClpP. *L. interrogans* also express two isoforms of ClpP (LepClpP1 and LepClpP2). These two isoforms together form the active tetradecameric barrel that exhibits proteolytic activity only in the presence of cognate chaperone LepClpX (Dhara et al. 2019). Moreover, the effect of ADEPs on LepClpP isoforms was quite intriguing in that the antibiotic can modulate LepClpP in an unconventional mechanism (Dhara et al. 2021). To further explore the functional activities of the ClpP isoforms, we decided to engineer site-directed LepClpP1 and LepClpP2 mutants. We wanted to test the activation property induced by these mutations at the crucial hotspots of LepClpP isoforms. The presence or absence of these hotspots may indicate how uncharacterized ClpP isoforms can be expected to function.

Pathogens possess many virulence factors, and delineating and understanding these determinants' function is paramount in understanding the pathogenesis of the diseases they cause. These factors' combined effect enables microbes to efficiently invade and colonize various tissue niches, obtain nutrients, and evade and suppress the host's immune response. In contrast to other pathogens, where experimental genetic inquiry has defined a set of virulence factors, the mechanisms by which pathogenic *Leptospira* cause disease remain largely unknown; mostly due to the recalcitrance of pathogenic *Leptospira* to genetic manipulation (Lehmann et al. 2014). There have been reports of site-directed homologous recombination being used for the deletion of chromosomal genes; however, these reports are exceptions rather than the norm (Dong et al. 2017). As targeted knockouts remain difficult, genetic manipulation of pathogenic *Leptospira* has been limited to random transposon-based mutagenesis in two of the more virulent serovars Manilae (Lourdault et al. 2011, Murray et al. 2010) and Lai (Bourhy et al. 2005). As ClpP is proved to be related to the virulence of most studied organisms as well as the survival of *M. tuberculosis* (harboring two ClpP isoforms like *Leptospira*) (Lavey et al. 2018) we were interested to genetically manipulate the saprophytic *L. biflexa* by knocking down one of the *clpP* isoforms and evaluate the functional and phenotypic changes in the mutant strain as a proof-of-principle.

4.3 RESULTS

4.3.1 Multiple sequence alignment of ClpP orthologs of pathogenic bacteria show conserved critical motifs required for ClpP activation

The primary sequences of *Leptospira* ClpP1 and ClpP2 were aligned to well-characterized ClpP orthologs in other pathogenic organisms. Multiple sequence alignment (MSA) of ClpPs in *Leptospira* and its orthologs in *Clostridium*, *Helicobacter, Listeria, Mycobacterium, Staphylococcus, Chlamydia, Pseudomonas, Salmonella, Streptococcus,* and *Escherichia* demonstrated to have conserved catalytic triad (Ser-His-Asp), an essential motif for the charge-relay in serine peptidase family (**Figure 4.1**). The catalytic triad residues in ClpP1 and ClpP2 of *Leptospira* achieved alignment at Ser98-His123-Asn172 and Ser97-His122-Asp173, respectively. Interestingly, ClpP1 isoforms of *Leptospira* have Asn172 residue instead of the commonly observed Asp residue in the catalytic triad, which is similar to that reported ClpP1 catalytic triad in *Listeria* (Zeiler et al. 2013) (**Figure 4.1**). Other critical hot spots of ClpP, like the Tyr activation trigger, Asp (Glu)/Arg oligomerization sensor domains, and the Gly-rich heptamer dimerization domain, are highly conserved in both ClpP isoforms of *L. interrogans.* Recently, a mutation in Tyr63 to Ala residue in *S. aureus* ClpP (StaClpP) led to a gain of function in the StaClpP^{Y63A} that was comparable to the dysregulation activity of WT StaClpP that occurs upon ADEPs treatment (Ni et al. 2016).

In contrast, significant variations are observed in the motif E-helix (α5), and the N-terminal loop of ClpP isoforms known to intercalate with the cognate opposite heptameric ring and its hexameric ATPase chaperone, respectively. Such amino acid sequence variations at these hotspots encouraged us to characterize the ClpP isoforms function by mutating specific conserved residues. The sequence identity of ClpP1 and ClpP2 amino acid of *Leptospira* with the orthologs in selected pathogenic bacteria is 35-66% and 35-50%, respectively (**Table 4.1**). The highest sequence identity of ClpP1 and ClpP2 amino acid of *Leptospira* was found with its orthologs in *Clostridium difficile* (66%) and *Streptococcus pneumoniae* (50%), respectively.

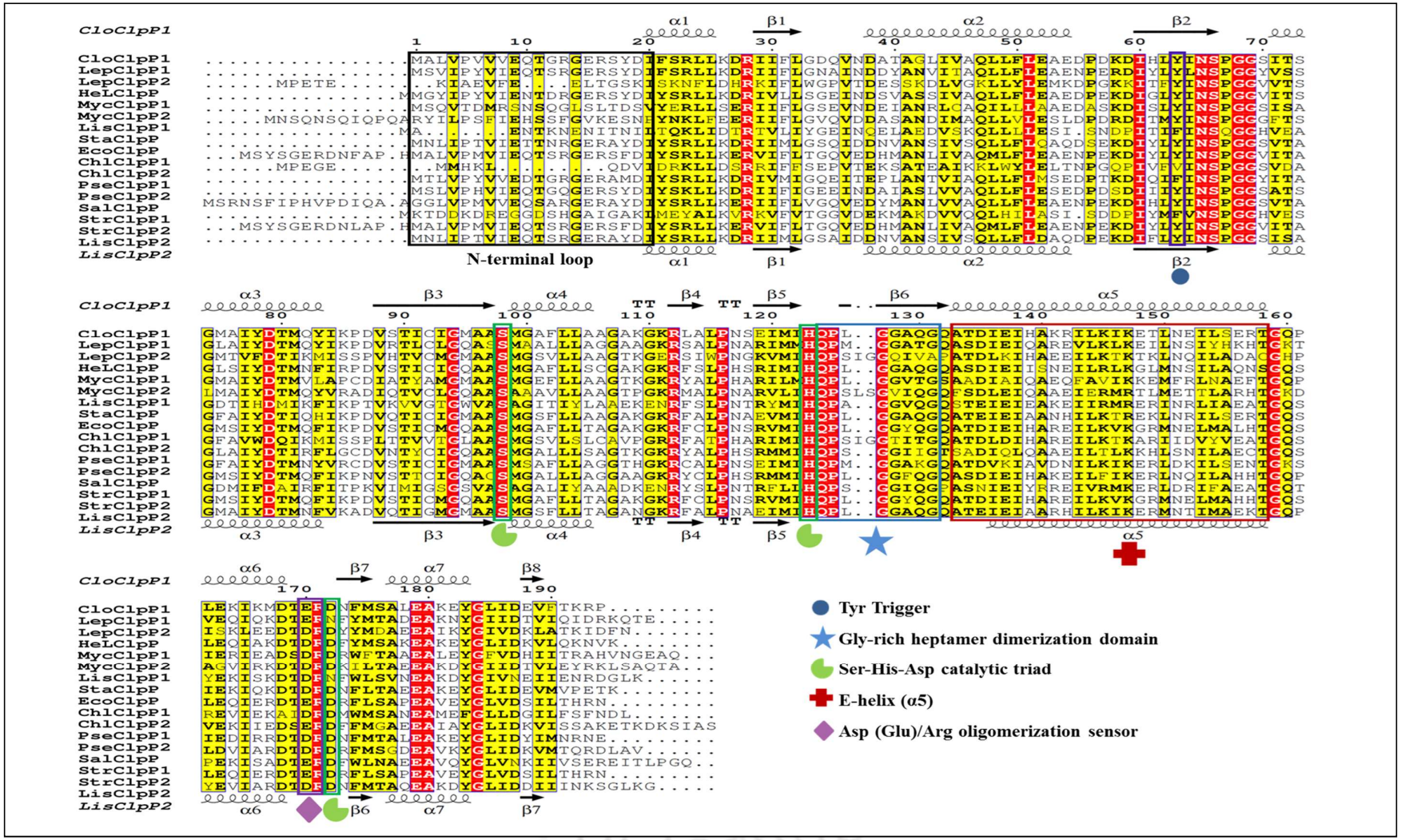
CloClpP1
LepClpP1
LepClpP2
HeLClpP
MycClpP1
MycClpP2
LisClpP1
StaClpP
EcoClpP
ChlClpP1
ChlClpP2
PseClpP1
PseClpP2
SalClpP
StrClpP1
StrClpP2
LisClpP2
LisClpP2
N-terminal loop
α1
β1
α2
β2
α3
β3
α4
β4
β5
β6
α5
α6
β7
α7
β8
Tyr Trigger
Gly-rich heptamer dimerization domain
Ser-His-Asp catalytic triad
E-helix (α5)
Asp (Glu)/Arg oligomerization sensor

Figure 4. 1. Multiple sequence alignment and secondary structure assignment of ClpP proteases Multiple sequence alignment of ClpP orthologs from various pathogenic bacteria was performed using Clustal Omega software. The ClpP orthologs used for amino acid alignment are CloClpP1: *Clostridium difficile* (Q180F0), LepClpP1: *Leptospira interrogans* Copenhageni (Q72SG6), LepClpP2: *L. interrogans Copenhageni* (Q72R01), HelClpP: *Helicobacter pylori* (P56156) MycClpP1: *Mycobacterium tuberculosis* (P9WPC5), MycClpP2: *M. tuberculosis* (P9WPC3), LisClpP1: *Listeria monocytogenes* (Q8Y7Y1), StaClpP: *Staphylococcus aureus* (P63786); EcoClpP: *Escherichia coli* (P0A6G7), ChlClpP1: *Chlamydia trachomatis* (P38002), ChlClpP2: *C. trachomatis* (084712), CloClpP2: *C. difficile* (Q180J6), PseClpP1: *Pseudomonas aeruginosa* (Q9I2U1), PseClpP2: *P. aeruginosa* (Q9HYR9), SalClpP: *Salmonella typhimurium* (P0A1D7), LisClpP2: *L. monocytogenes* (Q9RQI6). The UniProt accession numbers are provided in parenthesis. Secondary structural elements present in the CloClpP1 (PDB ID: 6mx2) and LisClpP2 (PDB ID: 4jct) structure are shown on the top and bottom of the sequence alignment, respectively. Identical and semi-identical residues in ClpP proteases are highlighted in red and yellow colors, respectively, with residue numbers at the top of the alignment after CloClpP1. The conserved key structural motifs in the ClpP isoforms, including the Tyr63 activation trigger, catalytic triad (Ser-His-Asp), Asp(Glu)/Arg oligomerization sensor domains, E-helix, and the Gly-rich heptamer dimerization domain, are outlined in different colored boxes and marked with specific symbols at the bottom of the box.

Table 4. 1**. Comparative analyses of *Leptospira* ClpP orthologs in selective pathogenic bacteria**

		***Leptospira interrogans* serovar Copenhageni**		***Mycobacterium tuberculosis***		***Listeria monocytogenes***		***Streptococcus pneumoniae***		***Pseudomonas aeruginosa***		***Escherichia coli***	***Staphylococcus aureus***	***Salmonella typhimurium***	***Helicobacter pylori***	***Clostridium difficile***		***Chlamydia trachomatis***	
Protein name (UniProt ID)		LepClpP1 (Q72SG6)	LepClpP2 (Q72R01)	MycClpP1 (P9WPC5)	MycClpP2 (P9WPC3)	LisClpP1 (Q8Y7Y1)	LisClpP2 (Q9RQI6)	StrClpP1 (A0A064BX72)	StrClpP2 (A0A0E9GPP2)	PseClpP1 (Q9I2U1)	PseClpP2 (Q9HYR9)	EcoClpP (P0A6G7)	StaClpP (P63786)	SalClpP (P0A1D7)	HelClpP (P56156)	CloClpP1 (Q180F0)	CloClpP2 (Q180J6)	ChlClpP1 (P38002)	ChlClpP2 (084712)
Percent sequence identity (Percent query coverage)	LepClpP1	100 (100)	42 (84)	49 (93)	51 (95)	35 (91)	62 (95)	52 (94)	60 (95)	62 (94)	40 (87)	61 (95)	62 (95)	61 (95)	64 (94)	66 (95)	60 (96)	39 (84)	55 (95)
	LepClpP2	42 (84)	100 (100)	41 (92)	46 (84)	35 (96)	44 (87)	40 (90)	50 (95)	48 (98)	39 (88)	48 (88)	49 (94)	48 (88)	45 (90)	50 (88)	43 (88)	50 (86)	43 (87)

4.3.2 Homology modeling of *Leptospira* ClpP isoforms and the selection of ClpP variants

Based on the MSA of the ClpP orthologs, the binding motifs of ClpP isoforms crucial for ClpP activation exhibited to be highly conserved. Among these hotspot regions, we decided to mutate some residues in each of the LepClpP isoforms based on the literature review (**Table 4.2**). The tertiary structure models of the ClpP1 and ClpP2 of *Leptospira* were generated based on the highest sequence identity with the respective ClpP orthologs (**Figure 4.2**).

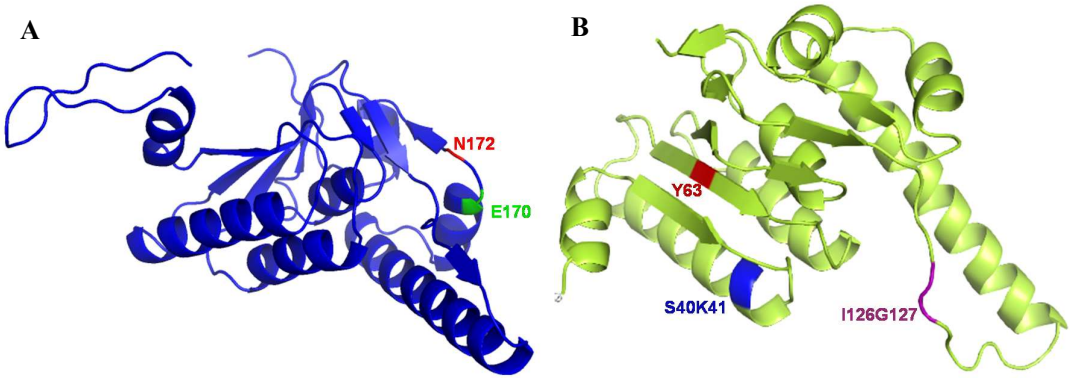

Figure 4. 2 Homology modelling of the ClpP1 and ClpP2 of *Leptospira*.
The cartoonic representation of the model ClpP1 **(A)** and ClpP2 **(B)** monomers from *Leptospira interrogans*. The ClpP1 and ClpP2 monomers are represented in marine and limon color, respectively. The ClpP1 was modelled based on known crystal structure of *Clostridium difficile* ClpP1 (CldClpP1, PDB id: 6mx2) as LepClpP1 has the highest sequence identity of 66% with CldClpP1 among all the compared ClpP orthologs. The ClpP2 was modelled based on known crystal structure of *S. pneumoniae* ClpP ($SpClpP^{A153P}$, PDB id: 17yo) as LepClpP2 has the highest sequence identity of 50% with SpClpP among all the compared ClpP orthologs. The residues to be mutated in LepClpP1 and LepClpP2 monomers are shown in different colors; Asn172 (red) and Glu170 (green) in ClpP1; Tyr63 (firebrick), Ser40Lys41 (blue), and Ile126Gly127 (purple) in ClpP2.

Table 4. 2 List of mutants of the leptospiral ClpP isoforms selected in this study at conserved ClpP hotspots

ClpP isoform (wild type variant)	Mutation	Conserved region	ClpP mutant
ClpP1	N172D	Catalytic triad	$ClpP1^{N172D}$
ClpP1	E170D	Oligomerization sensor residue	$ClpP1^{E170D}$
ClpP2	S40AK41N	Hydrophobic region in contact with N-terminal β-hairpin	$ClpP2^{S40AK41N}$
ClpP2	Y63A	Tyr activation trigger	$ClpP2^{Y63A}$
ClpP2	I126G127_del	Gly-rich heptamer dimerization domain	$ClpP2^{I126G127\_del}$

4.3.3 Purification of leptospiral ClpP isoforms variants

The site-directed mutagenesis was conducted individually in pET23a-*clpP1* and pET23a-*clpP2* expression vectors. The plasmid sequencing results suggested all the plasmids were substituted correctly as per the primer designed for site-directed mutagenesis except the one expressing LepClpP1^{E170D}. The purification of the four mutant variants of ClpP1 and ClpP2 (LepClpP1^{N172D}, LepClpP2^{S40AK41N}, LepClpP2^{Y63A}, LepClpP2^{I126G127_del}) using the Ni-NTA affinity column chromatography was conducted, as described before for the pure LepClpP isoforms (**Figure 4.3**). The double residue deletion mutant of LepClpP2 at Ile126 and Gly127 positions is denoted as LepClpP2^{I126G127_del}, where 'del' in the superscript denotes deletion.

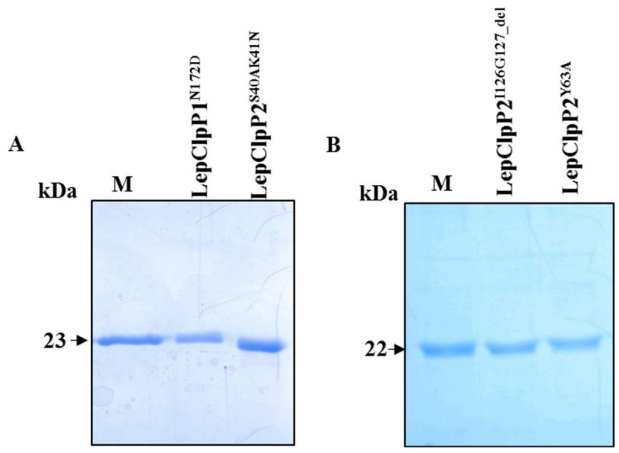

Figure 4. 3 Purification of leptospiral ClpP isoforms variants.
The affinity purified *Leptospira* recombinant ClpP mutants. **(A)** The Ni-NTA affinity purified LepClpP1^{N172D} (~ 23 kDa), LepClpP2^{S40AK41N}(~ 22 kDa) and **(B)** The Ni-NTA affinity purified LepClpP2^{I126G127_del} and LepClpP2^{Y63A} (~ 22 kDa) proteins were resolved on 12% SDS-polyacrylamide gel and stained with Coomassie-blue. M denote the marker of size 23 kDa and 22 kDa for **(A)** and **(B)** respectively.

4.3.4 Oligomerization of leptospiral ClpP variants

The pure LepClpP isoforms and their mutant variants were resolved on a native polyacrylamide gel electrophoresis (Native-PAGE) and stained with Coomassie blue to determine any change in the oligomerization property (**Figure 4.4**). The pure LepClpP1^{N172D} mutant was resolved at a higher oligomeric species than its corresponding LepClpP1 (~ 480 kDa, 21-mer). There was a visible difference in the oligomerization of the LepClpP2^{S40AK41N} mutant versus the LepClpP2. Few proportions of this LepClpP2^{S40AK41N} mutant variants were resolved in the tetradecameric size (~308 kDa), while the significant fraction was determined in the heptameric size (~154 kDa). Among all, the LepClpP2^{I126G127_del} mutant was resolved at the tetradecameric size (~308 kDa), like its corresponding pure LepClpP2.

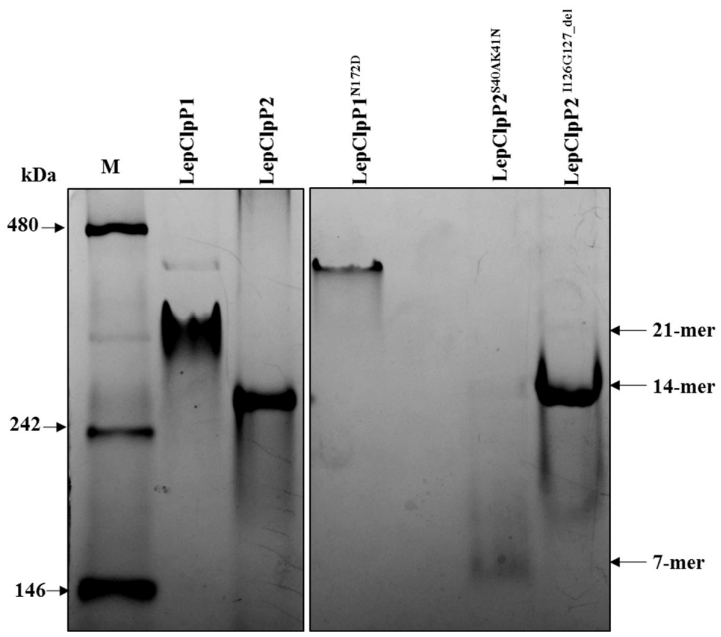

Figure 4. 4 Oligomerization of *Leptospiral* ClpP isoforms variants.
Pure LepClpP isoforms and their mutants were resolved on a 7.5% native-PAGE. The standard molecular weight was run in lane 1 (M). The purified LepClpP1 and LepClpP2 resolved at 21- and 14-mer respectively, while LepClpP1^{N172D} resolved higher than 21-mer. The LepClpP2^{S40AK41N} was resolved at 14-mer and 7-mer. The LepClpP2^{I126G127_del} resolved at 14-mer. The native gel was stained with Coomassie blue and for clarity the image represented here is obtained after inversion.

4.3.5 Leptospiral ClpP isoforms variants exhibited a gain of functional activity

The mutation of ClpP residues involved in functional activation has recorded a modification in ClpP's activity in different organisms like *Listeria*, *Staphylococcus*, and *Bacillus* (Zeiler et al. 2013, Ni et al. 2016, Lee et al. 2010). Therefore, the LepClpP1 and LepClpP2 variants' peptidase activity of *Leptospira* was evaluated using the fluorogenic dipeptide model substrate Suc-LY-AMC (S1) (**Figure 4.5**). None of the pure LepClpP mutants or their derived mixtures were enzymatically active, measured till one h of the peptidase reaction. For further validation, the peptidase reaction duration was extended until 24 h. Interestingly, one of the mutant heterocomplex LepClpP1^{N172D}P2 had a 29% gain in the peptidase activity compared to the control (buffer and substrate), while the pure LepClpP1^{N172D} remained inactive during 24 h reaction period. To our surprise, one of the pure LepClpP2^{S40AK41N} with a mutation at two residues exhibited a 12% gain in the peptidase activity than the control, while the heterocomplex LepClpP1P2^{S40AK41N} exhibited an 18.5% gain. In contrast, neither pure LepClpP2^{I126G127_del} nor the LepClpP1P2^{I126G127_del} mixture was enzymatically active.

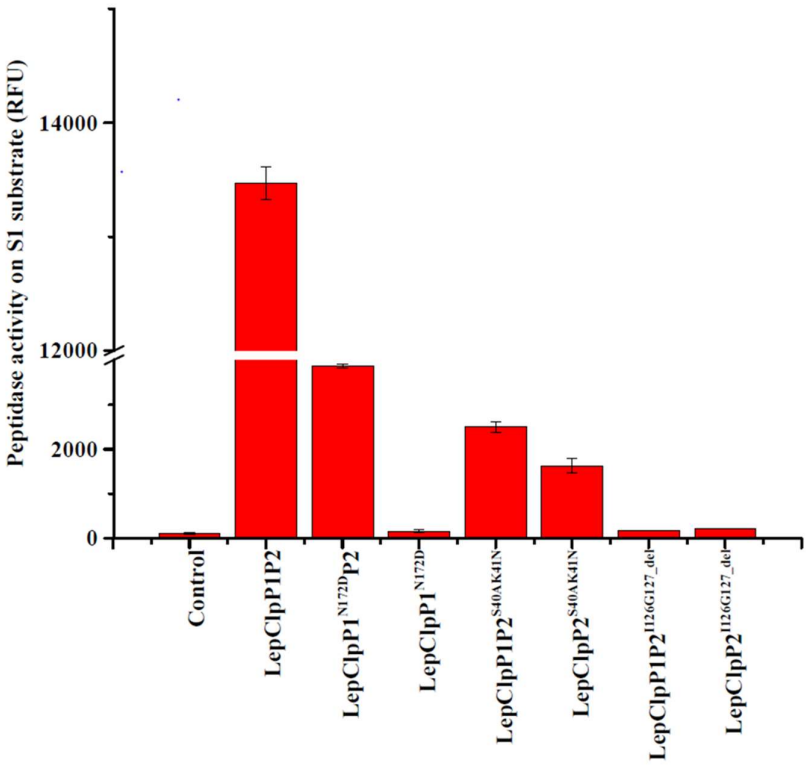

Figure 4. 5 Peptidase activity of the *Leptospiral* ClpP isoforms variants.
Peptidase activity of the LepClpP1 and LepClpP2 mutants measured as the end-point fluorescenece after 24 h of enzymatic reaction. The control shows the background fluorescence of the substrate in the reaction. As expected the wild-type LepClpP1P2 has the highest peptidase activity compared to the other mutant LepClpP isoforms. The LepClpP mutant isoforms exhibited varied peptidase activities. The error bars represent the standard errors of mean (SEM) from the two independent experiments performed.

A recent study revealed that the mutation of Tyr63 of StaClpP leads to gain-of-function in peptides activity versus the StaClpP (Ni et al. 2016) through "open-gate" activated StaClpP peptidase form without the cognate chaperone or ADEP activators. Thus, as a proof-of-concept, the proteolysis of Tyr63 mutation in LepClpP2 in the absence of ATPase chaperones or ADEP activators was investigated. As anticipated, the pure LepClpP isoforms and LepClpP1P2 were not enzymatically active against labeled FITC-casein in the absence of cognate ATPase (**Figure 4.6A**). However, the mutant heterocomplex LepClpP1P2^{Y63A} and even pure LepClpP2^{Y63A} could degrade the unlabelled β-casein and labeled FITC-casein in the absence of its physiological chaperone under the given *in vitro* condition (**Figure 4.6**).

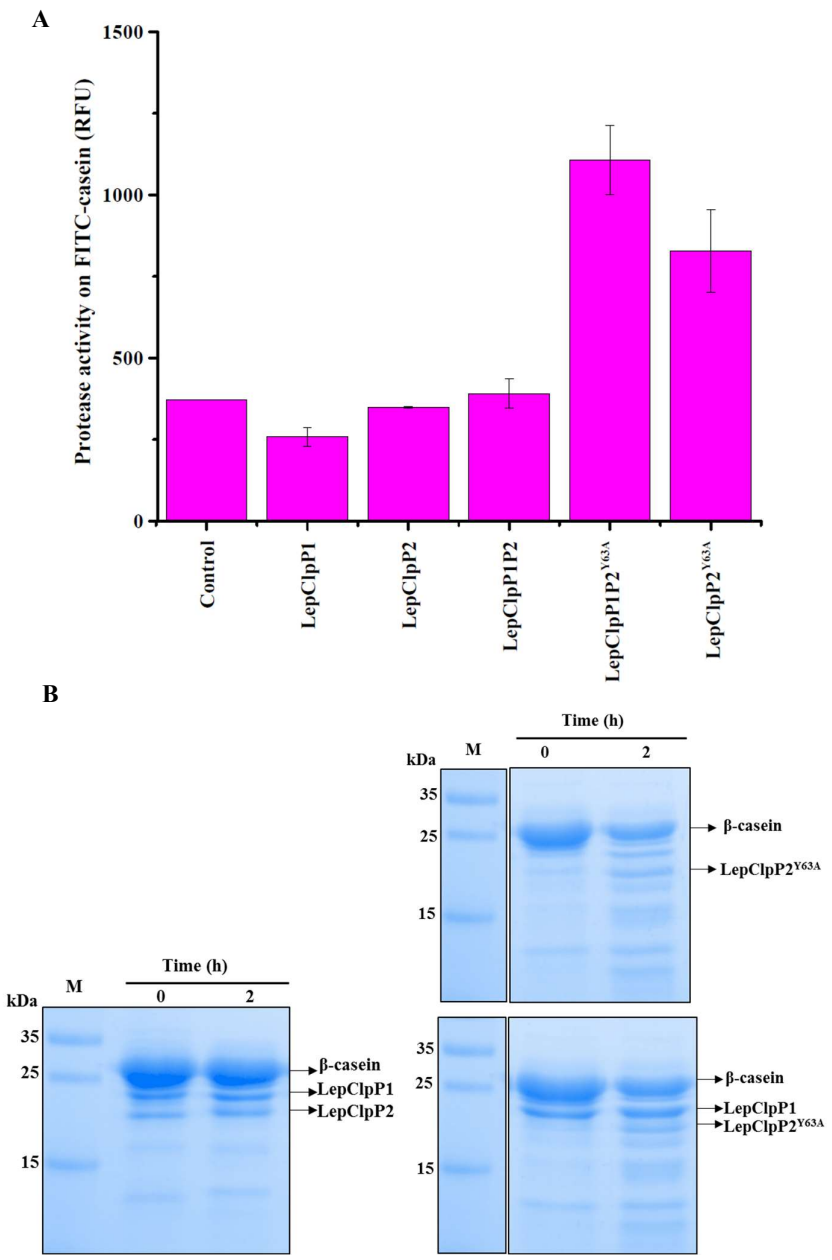

Figure 4. 6 Protease activity of the leptospiral ClpP2^{Y63A}.
(A) Protease activity of the LepClpP1 and LepClpP2 mutants on FITC-casein measured as the end-point fluorescenece after 2 h of enzymatic reaction. The control shows the background fluorescence of the substrate in the reaction. The pure LepClpP1, LepClpP2 and LepClpP1P2 did not show any activity compared to the control, while the LepClpP2^{Y63A} and LepClpP1P2^{Y63A} were able to degrade FITC-casein at variable rates. The higher activity of LepClpP1P2^{Y63A} heterocomplex is possiby due to the presence of pure rClpP1. The error represent the standard deviation (SD) from a single experiment performed in duplicate. **(B)** Denaturing gel electrophoresis showing the activity of pure LepClpP isoforms (left panel) and LepClpP2^{Y63A}, LepClpP1P2^{Y63A} (right panel) on the β-casein substrate. There is a visible degradation of casein by LepClpP2^{Y63A} and LepClpP1P2^{Y63A}.

The differential functional activities of LepClpP mutant isoforms encourage further studies that can disclose remarkable activation of *Leptospiral* ClpP.

4.3.6 Caseinolytic protease genes in the *Leptospira biflexa* genome

The genus *Leptospira* comprises both saprophytic and pathogenic spirochetes, such as *L. biflexa* and *L. interrogans* (Faine 1994). The ignorance of spirochetal biology is majorly due to the lack of methods available for genetic analyses of these organisms. The genetic modifications of pathogenic *Leptospira* are limited to random transposon mutagenesis, and there are only a few examples of mutants obtained by targeted mutagenesis (Picardeau 2015, Murray 2015). But in recent years, saprophytic *L. biflexa* has been utilized as a model bacterium among spirochetes for their ease in targeted mutagenesis (Louvel and Picardeau 2007). Thus, we decided to inactivate one of the *clpP* homologs of *L. biflexa* strain Patoc I by double homologous recombination as a first step to elucidate the function of the caseinolytic protease (Clp) system in *Leptospira*.

We first identified the orthologous *clp* genes in *L. biflexa* (**Figure 4.7**) and compared them with the *clp* genes of pathogenic *L. interrogans* (**Table 4.3**). The genome analysis of the sequenced spirochete *L. biflexa* serovar Patoc strain Patoc I (Paris) shows that it harbors various genes of the *clp* system, similar to that of *L. interrogans*, but with some exceptions. The *clp* system in *Leptospira* is composed of genes encoding catalytic, regulatory, and adaptor proteins. The set of genes encoding catalytic components are *clpP1* (*LEPBI_I1760*) and *clpP2* (*LEPBI_I0969*). The Clp AAA+ (ATPases associated with various cellular activities) are encoded by *clpX (LEPBI_I0970)*, *clpA (LEPBI_I1852),* and *clpC (LEPBI_I2574)* genes, whereas the adaptor proteins are encoded by *clpS* (*LEPBI_I1853, LEPBI_I2410,* and *LEPBI_I2966)*. However, the number of *clp* genes in *L. biflexa* is lower than pathogenic *L. interrogans* indicating tighter regulation of the Clp system for the survival of pathogens in diverse hosts and environments. For instance, the catalytic unit ClpQ, a threonine protease of *L. interrogans,* and the interacting chaperone ClpY forming the functional protease ClpYQ (also known as HslUV) (Dong et al. 2017) are in opposite orientation in *L. biflexa* compared to *L. interrogans* (**Figure 4.7**). The genome of *L. biflexa* lacks class-II chaperone ClpB among the Clp-ATPases of the Clp system. The existence of three paralogs of *clpS* genes was also predicted in *L. biflexa*, in contrast to two *clpS* paralogs in *L. interrogans*. It also supports the fact that leptospires have many novel pathogenesis mechanisms that are yet to be identified.

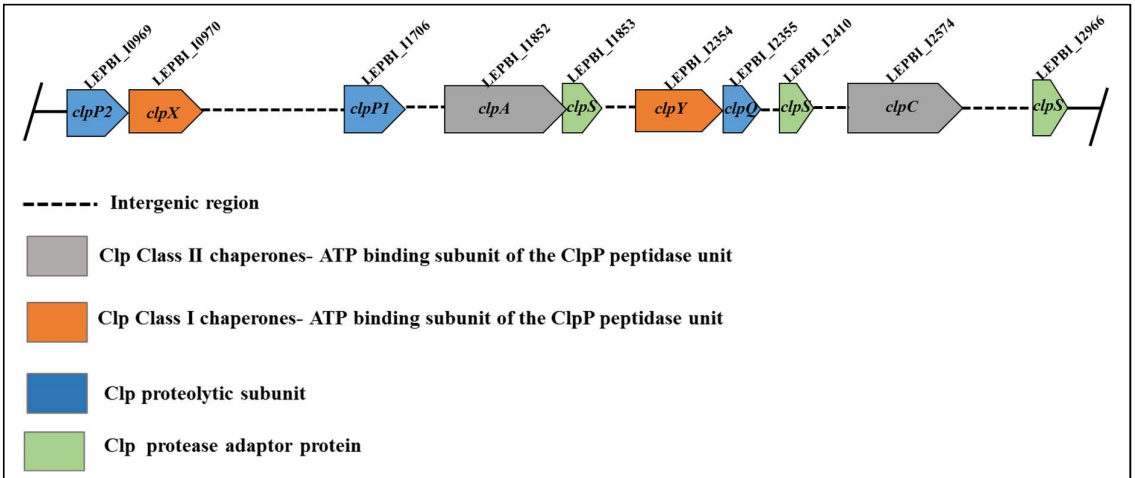

Figure 4. 7 Schematic representation of genes encoding caseinolytic protease system (*clp*) in *L. biflexa* genome.

The arrangement of *clp* genes and its annotation in *Leptospira* genome are represented in an arrow diagram (not scaled) based on bioinformatics analysis. Caseinolytic protease system in *L. biflexa* is composed of genes encoding catalytic subunits (*clpP1, clpP2, clpQ*; blue color), Clp AAA+ subunits (*clpA*, *clpC*, *clpX,* and *clpY*), and adaptor proteins (*clpS*; green color). The intergenic regions are depicted as interrupted lines. The Clp AAA+ proteins have been grouped as class-I (ClpX; orange color) and as the class-II chaperone (ClpA, ClpC; grey color) based on the number of ATPase domain(s).

Table 4. 3. Comparative analyses of the gene sequence identities of the *clp* genes of pathogenic *L. interrogans* and saprophytic *L. biflexa*

Leptospira species	Locus	Gene	No. of bases	No. of identical bases/total (% identity)
L. interrogans	LIC11951	*clpP2*	594	370/682 (54)
L. biflexa	LEPBI_I0969		603	
L. interrogans	LIC11417	*clpP1*	597	351/715 (49)
L. biflexa	LEPBI_I1760		609	
L. interrogans	LIC11418	*clpX*	1263	888/1351 (65)
L. biflexa	LEPBI_I0970		1287	
L. interrogans	LIC11814	*clpA*	2223	1415/2310 (61)
L. biflexa	LEPBI_I1852		2271	
L. interrogans	LIC10339	*clpC*	2541	1824/2574 (71)
L. biflexa	LEPBI_I2574		2550	
L. interrogans	LIC11815	*clpS*	336	216/353 (61)
L. biflexa	LEPBI_I1853		342	
L. interrogans	LIC11815	*clpS*	336	165/351 (47)
L. biflexa	LEPBI_I2410		285	
L. interrogans	LIC11356	*clpS*	321	220/333 (66)
L. biflexa	LEPBI_I2966		327	
L. interrogans	LIC11600	*clpQ (HslV)*	543	388/571 (68)
L. biflexa	LEPBI_I2355		534	
L. interrogans	LIC11601	*clpY (HslU)*	1440	939/1472 (64)
L. biflexa	LEPBI_I2354		1419	

4.3.7 Generation of *L. biflexa clpP2* deletion mutant

For the Δ*clpP2* mutant generation by homologous recombination, the first step was to construct a suicidal plasmid containing the flanking regions (1000 nt) required to knock down the gene of interest *LEPBI_I0969*, a *clpP2* homolog of *Leptospira biflexa*. The 1000 bp flanking the upstream (P1P2) and downstream region (P3P4) to *LEPBI_I0969* open reading frame were amplified by PCR using the genomic DNA of *L. biflexa* strain Patoc1 (**Figure 4.9 A**). The flanking DNA regions were then inserted in the previously constructed mutagenesis plasmid pMutLept (**Figure 4.8 A**) to generate the suicidal pMutLept$^{5'\text{-P1P2-pflaBKan-P3P4-3'}}$ (**Figure 4.8 B and 4.9 B-E**). The Amp^{R} marker of the pMutLept$^{5'\text{-P1P2-pflaBKan-P3P4-3'}}$ was inactivated before electro-transformation of competent spirochetes (**Figure 4.8 F-G**).

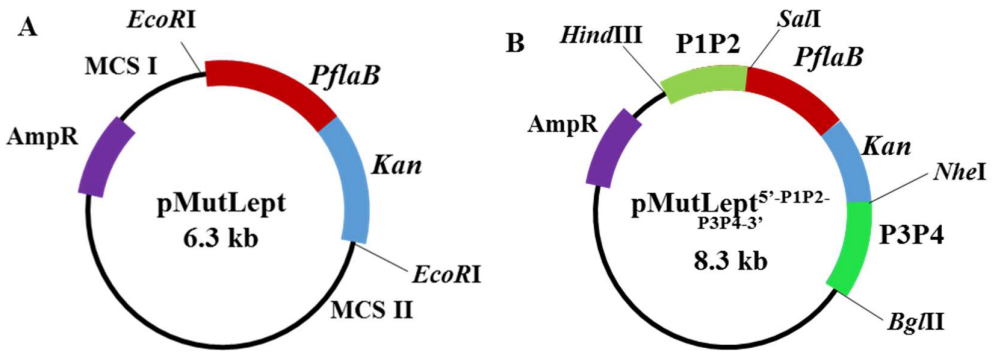

Figure 4. 8 Schematic representation of mutagenesis plasmids for knocking down the *L. biflexa clpP2* gene *(LEPBI_I0969)*.
(A) The mutagenesis plasmid constructed previously in lab, to be used for the generation of suicaidal plasmid. The plasmid named pMutLept, has an incorporation of *Leptospira flaB* promoter driven by a kanamycin (Kan) cassette. There are two multiple cloning sites (MCS I and MCS II) that will be used to insert the amplified 1000 bp upstream and downstream regions flanking the gene of interest, here *L. biflexa clpP2*, to knock down. **(B)** The generated suicaidal plasmid pMutLept$^{5'\text{-P1P2-pflaBKan-P3P4-3'}}$ for knocking down the *L. biflexa clpP2*. The 1000 bp flanking the upstream (P1P2) region to *LEPBI_I0969* (*clpP2*) open reading frame is inserted in the MCS I between the *Hind*III and *Sal*I restriction digestion sites and 1000 bp flanking the downstream region (P3P4) to *LEPBI_I0969* (*clpP2*) open reading frame is inserted in the MCS II between the *Nhe*I and *Bgl*II restriction digestion sites.

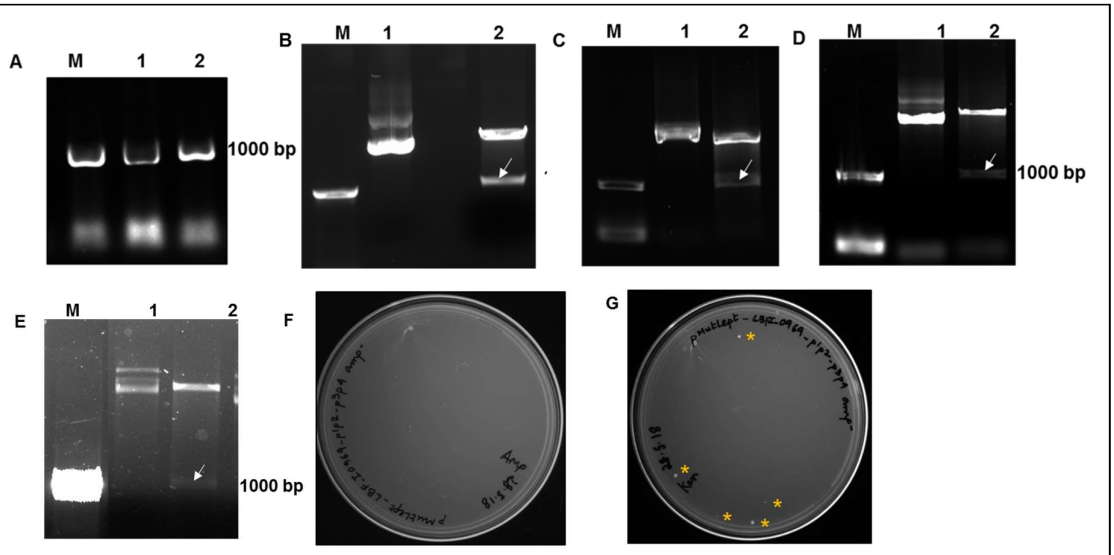

Figure 4. 9 Insertion of upstream and downstream flanking regions in the mutagenesis plasmid pMutLept for *clpP2* gene knockdown in *L. biflexa* and inactivation of the Ampicillin marker of the pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$

(A) PCR amplification of 1000 bp upstream and downstream flanking regions of *clpP2*. M: Marker of 1000 bp, 1: Amplified 1000 bp upstream flanking region (P1P2) and 2: Amplified 1000 bp downstream flanking region (P3P4). **(B)** Restriction digestion of TA-P1P2 by *Hind*III and *Sal*I. M: Marker of 1000 bp, 1: Undigested TA-P1P2, 2: Digestion of cloned P1P2 from pTZ57R/T. **(C)** Restriction digestion of TA-P3P4 by *Nhe*I and *Bgl*II. M: Marker of 1000 bp, 1: Undigested TA-P3P4, 2: Digestion of cloned P3P4 from pTZ57R/T. (TA- pTZ57R/T) **(D)** Restriction digestion of pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan}$ by *Hind*III and *Sal*I to confirm insertion of upstream flanking region inside the mutagenesis plasmid pMutLept. M: Marker of 1000 bp, 1: Undigested pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan}$, 2: Digestion of cloned P1P2 from pMutLept. **(E)** Restriction digestion of pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$ by *Nhe*I and *Bgl*II to confirm insertion of downstream flanking region inside the mutagenesis plasmid pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan}$. M: Marker of 1000 bp, 1: Undigested pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$, 2: Digestion of cloned p3p4 from pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$. The gene fragment fall-outs of the digested plasmids are marked by white arrows. **(F and G)** Ampicillin marker in pMutLept$^{5'P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$ was inactivated by site directed mutagenesis. The Amp inactivated plasmid was transformed *in E. coli* DH5α cells and grown overnight in Ampicillin-Amp (**Figure F**) andKanamycin-Kan plates (**Figure G**) separately. There were no visible colonies on Amp plate, and only 5 colonies (denoted by yellow asterisk) on Kan plate.

The plasmid DNA from the transformed *E. coli* DH5α cells was extracted, and electroporation was carried out in the competent wild-type *L. biflexa* strain Patoc1. However, even after multiple transformation attempts, we were unsuccessful in isolating any *L. biflexa* Δ*clpP2* mutant. In fact, no clones of any *Leptospira* were seen on the plate after transformation with the constructed suicidal plasmid pMutLept$^{5'\text{-}P1P2\text{-}pflaBKan\text{-}P3P4\text{-}3'}$. One of the speculations for such observation of no transformants could be the co-localization of *clpP2/clpX* (**Figure 4.7**) that could form an operon suggesting the essentiality of *clpP2* or *clpX.* On the other hand, clones was seen when competent spirochete transformed with empty suicidal plasmids when grown in plates without antibiotic kanamycin, suggesting the essentially of ClpP for spirochete survival or can be possible due to unknown technical issues during transformation protocol.

4.4 DISCUSSION

Although most organisms possess a single ClpP protein with a conserved fold (Wang et al. 1997, Geiger et al. 2011, El Bakkouri et al. 2010, Gribun et al. 2005, Kang et al. 2004, Lee et al. 2010), the genomes of some organisms encode two or more ClpP isoforms (Akopian et al. 2012, Zeiler et al. 2011, Hall et al. 2016, Dhara et al. 2019, Pan et al. 2019, Schelin et al. 2002, Stanne et al. 2007, Viala and Mazodier 2002, Viala et al. 2000). For instance, in a cyanobacterial system, the ClpP heptamers have a mixed composition that interacts with different chaperones (Stanne et al. 2007). Contrary to that, ClpPs from firmicutes *L. monocytogenes* (LisClpP1 and LisClpP2), and *M. tuberculosis* (MycClpP1 and MycClpP2); bacteria *C. trachomatis* (ChlClpP1 and ChlClpP2), and *L. interrogans* (LepClpP1 and LepClpP2) have been found to assemble into heterooligomeric complexes composed of two homoheptamers (Zeiler et al. 2011, Akopian et al. 2012, Pan et al. 2019, Dhara et al. 2019, Wood et al. 2020). Inhibition of LisClpP2 with lactone based inhibitors led to downregulation of virulence without affecting the viability (Böttcher and Sieber 2009). In contrast, both MycClpP1 and MycClpP2 are essential for bacterial survival (Raju et al. 2012). The catalytically inactive ChlClpP2 significantly impacted developmental cycle progression by reducing the overall number of organisms, further emphasizing the defined functional roles of ClpPs among species.

The MSA of ClpP orthologs of pathogenic bacteria (**Figure 4.1**) reveals several characteristic features conserved among this protease class. A catalytic triad (Ser98-His123-Asp172) essential for proteolysis, a central E-helix with Gly-rich loop required for establishing contacts between the two heptamers, and an N-terminal region for interaction with an AAA+ chaperone/ATPase can be observed in all available crystal structures to date (Wang et al. 1997, Geiger et al. 2011, Gersch et al. 2012, El Bakkouri et al. 2010, Gribun et al. 2005, Ingvarsson et al. 2007, Kang et al. 2004, Lee et al. 2010, Felix et al. 2019, Kim and Kim 2008, Zeiler et al. 2013). For the activation of StaClpP, the enzymes switch their conformational states with an active and inactive catalytic triad corresponding to an extended and a bent E-helix, respectively (Geiger et al. 2011, Gersch et al. 2012). The highly conserved Asp170/Arg171 sensor residues link the StaClpP oligomerization with catalytic activity and bring about the compressed (inactive) to extended (active) states in the tetradecamer (Gersch et al. 2012). According to this model, a ClpP heptamer's sensor residues do not interact with their adjacent ring counterparts and have an inactive triad. In the tetradecameric state, the sensor feedbacks the correct assembly, thereby ensuring controlled proteolysis. ClpP gains its catalytic activity in complex with ATPases such as ClpX/ Clp A/ ClpC. These chaperones bind to the axial pores

of ClpP, unfold the misfolded/damaged protein, and pass it through the proteolytic chamber, housing the 14 active sites (Baker and Sauer 2012). The structural evidence (Glynn et al. 2009, Stinson et al. 2013, Bewley et al. 2006, Guo et al. 2002, Kim and Kim 2003, Szyk and Maurizi 2006) and biochemical analyses (Kim et al. 2001, Martin et al. 2007, Singh et al. 2001, Joshi et al. 2004, Gribun et al. 2005) reveal that major interactions occur between the IGF/L loops of Clp-ATPases and the hydrophobic pockets of ClpP proteases. These hydrophobic pockets surround the axial entrance-pore at the N-terminus and are located between the adjacent ClpP monomers. Cryo-electron microscopy (EM) has shown that EcoClp-ATPases binding enlarges the entrance pores for facilitating substrate entry (Effantin et al. 2010). Tyr63 activation trigger is a key component in the hydrophobic pocket that interacts with ATPases and ADEPs and initiates the dysfunctional activation of StaClpP toward the proteolysis of mature proteins (Ni et al. 2016). The mutational studies at these conserved motifs have revealed changes in the oligomeric and functional properties of the ClpP proteins (Zeiler et al. 2013, Geiger et al. 2011, Gersch et al. 2012, Leodolter et al. 2015, Gersch et al. 2015, Ni et al. 2016). Thus we were interested in mutating few residues in these conserved hotspots of LepClpP1 and LepClpP2 to study their oligomeric and biochemical characteristics. As a proof-of-concept, we initially decided to generate five LepClpP mutant isoforms (LepClpP1^{N172D}, LepClpP1^{E170D}, LepClpP2^{S40AK41N}, LepClpP2^{Y63A} and LepClpP2^{I126G127_del}). The rationality behind choosing these particular residues is discussed in the following sections.

Interestingly, there is a distinct difference between the two LepClpP homologs in their catalytic triad composition. An Asn172 residue in LepClpP1 replaces Asp172 of LepClpP2, an unusual observation within serine protease, also found in LisClpP1 (**Figure 4.1**). The catalytic triad redesign in LisClpP1 by an N172D mutation showed an ~20 fold increase in peptidase activity against fluorogenic dipeptide Suc-LY-AMC compared to the wild-type LisClpP1. The replacement of Asp with Asn significantly influences the strength of the catalytic charge relay system. It is argued that the strength of the hydrogen bonds is reduced within the active sites when Asp172 is replaced by Asn172, thereby reducing the nucleophilicity of Ser98 (Zeiler et al. 2013). However, the pure LepClpP1^{N172D} mutant was non-reactive, but after coupling with its counterpart wild-type LepClpP2, it became enzymatically active, albeit lesser than wild-type LepClpP1P2 (**Figure 4.5**). We expected a surge in LepClpP1^{N172D}P2 activity due to the supposed increase in the charge relay system's strength. Also, the LisClpP1^{N172D} formed a functional tetradecamer as opposed to the LisClpP1 that existed predominantly as an inactive heptamer (Zeiler et al. 2013). Similarly, the LepClpP1^{N172D} oligomerized at a larger size than 21-mer, i.e., more than the size of the LepClpP1. However, a very small fraction of wild-type

LepClpP1 was also oligomerized at the size of LepClpP1^{N172D} (**Figure 4.4**). These findings indicate that the active site mutation affects reactivity as well as the oligomerization of the protease.

The StaClpP activation model requires a large-scale conformational change from a compressed to an extended state by the rotation of the two heptameric rings with respect to each other by eight degrees around the axial direction (Geiger et al. 2011). The StaClpP transition poses the question of why the ring-ring association is not disrupted despite such conformational change. Detailed analysis of the StaClpP ring-ring interface revealed that helix α6 shifts upto 9Å, and the residues Asp170/Arg171 adjacent to the helix form salt bridges with their counterparts in the opposite ring (Geiger et al. 2011, Gersch et al. 2012). These salt bridges are rearranged but remain extended (Szyk and Maurizi 2006) and are prerequisites for stabilizing the ring-ring interface (Gribun et al. 2005). The stabilization of the ring-ring interface is brought about by this oligomerization sensor domain (Asp170/Arg171) that keep the StrClpP tetradecamer intact without dissociation in *Streptococcus pneumoniae* (Gribun et al. 2005) and a partially disordered handle domain consisting of central E-helix (α5) and β6 strand as observed in MycClpP1 (Ingvarsson et al. 2007). A close observation of LepClpP1 revealed that Glu170 in the oligomerization sensor domain replaces Asp170 of LepClpP2. Though this observation is noticed in quite a few ClpP orthologs (**Figure 4.1**), we wanted to mutate the Glu170 residue to the more conserved Asp residue to notice any difference in reactivity and oligomerization. However, we did not proceed with our functional studies because the mutation could not be confirmed after sequencing.

The next mutation is related to the Gly-rich loop of the handle region of the LepClpP2 monomer. Few conserved residues of the Gly-rich heptamerization domain form a part of the hinge region that underlies the functionally required conformational switch in the StaClpP handle region (Geiger et al. 2011). Mutations of Gly127, Gly131, along with Gly128 that form a part of the hinge region in StaClpP to Ala completely abolished the activity. (Geiger et al. 2011). Mutation of other residues in the hinge region, Glu135, and Leu144 either partially reduced or abolished the StaClpP activity due to oligomerization defects (Zeiler et al. 2011, Geiger et al. 2011). The findings established the need for the functionally essential switch that interconverts the two ClpP conformations. We observed that LepClpP2 has two extra residues Ile126 and Gly127, in the Gly-rich loop (β6 strand) (**Figure 4.1**). Thus, we decided to delete these two residues and analyze the effect of such mutations on the LepClpP function. The deletion of Ile126 and Gly127 did not result in the reactivity of pure LepClpP2^{I126G127_del} but surprisingly completely abolished the activity of the LepClpP1P2^{I126G127_del} heterocomplex

(**Figure 4.5**). However, the oligomerization property was not compromised, and pure LepClpP2^{I126G127_del} formed a tetradecamer like the wild-type LepClpP2 (**Figure 4.4**). Therefore, initial studies show that the deletion of Ile126 and Gly127 significantly impacts the LepClpP activation. It can be hypothesized that deleted residues are an essential part of the hinge region of LepClpP1P2 tetradecamer. Removing them renders the underlying conformational switch non-functional and stops it from participating in the transition of ClpP from inactive to the active state.

Next, we aimed to create a LepClpP2 variant with impaired chaperone binding by mutating a key component, Tyr63, in the hydrophobic pocket. The hydrophobic pockets or patches located in the cleft of the apical surfaces on the two adjacent ClpP monomers are the main interaction sites of the Clp-ATPase chaperones (Wang et al. 1997, Gribun et al. 2005, Schmitz et al. 2014). In most cases, where the ClpP protein is a heterocomplex composed of two heptameric rings of ClpP1 and ClpP2, the ClpP2 acts as a chaperone interaction platform (Leodolter et al. 2015, Pan et al. 2019, Gatsogiannis et al. 2019). Computational modeling also predicts the presence of hydrophobic pockets in the LepClpP2 (Dhara et al. 2020). So, we mutated the Tyr63 to Ala in LepClpP2. In the extended or compressed state of StaClpP, the side chains of Tyr63 have always been in a staggered conformation. The staggered conformation does not let the apo-ClpP achieve the activated 'open-gate' form due to the energy barrier. The binding of ADEPs to StaClpP provides that dynamic energy to overcome the transformation barrier, thereby activating the ClpP peptidase. Thus mutations in the hydrophobic pockets were created to check whether it could convert StaClpP into an uncontrollable protease capable of degrading unfolded proteins, as occurs in the presence of ADEPs. The mutated StaClpP^{Y63A} could efficiently degrade poorly folded β-casein and also had impaired protease activity (Ni et al. 2016). In contrast, the pure LepClpP2^{Y63A} variant could degrade poorly folded casein substrates without the aid of its counterpart wild-type LepClpP1 or the ClpX ATPase chaperone. The wild-type LepClpP1P2 cannot degrade the full-length casein protein without the cognate chaperone LepClpX or ADEP1 (Dhara et al. 2019, Dhara et al. 2020), whereas LepClpP2^{Y63A} can degrade the substrates (**Figure 4.6 A and B**). It can be hypothesized that LepClpP2^{Y63A} has overcome the need for ATPase or ADEP1 to degrade poorly folded proteins by overcoming the energy barrier. It will be engaging in future studies to know the reason for gain-of-function in the pure LepClpP2 mutant, whether the protease activity is compromised, and as reported in the literature, whether LepClpP2 is the binding site of ATPase chaperones or not in the LepClpP1P2 heterocomplex.

The last LepClpP mutant analyzed among the four generated mutants was LepClpP2^{S40AK41N}. We found that the residues Ser40 and Lys41 are in the α2 helix close to the N-terminal β-hairpin loop (β2 strand) in the LepClpP2 (**Figure 4.1**). We were surprised to observe that Ala40 and Asn41 in LepClpP1 were replaced by Ser40 and Lys41 in LepClpP2. This was an unusual observation as residues at these two positions were either conserved or semi-conserved in all other ClpP orthologs. Thus we were interested in redesigning these residues in LepClpP2 to S40AK41N to match with LepClpP1 and characterize the generated LepClpP2 mutant. Based on the positions of Ser40 and Lys41, they seem to reside in the hydrophobic cluster near the N-terminal β-hairpin region of LepClpP2. The *B. subtilis* ClpP (BacClpP) activation is triggered by the structural transition from a closed to an open-pore state when ADEP binding brings about local changes in the interaction sites (Lee et al. 2010). The structural examination of BacClpP, N-terminal β-hairpin regions revealed that hydrophobic residues Pro4 and Val6 of the first β1 strand of one BacClpP heptameric subunit interacted with Leu24 and Phe49 from helices α1 and α2 from the adjacent subunit. The hydrophobic cluster formation by the two ClpP subunits seems to be crucial for the BacClpP activation. The interaction between the N-terminal segment and the ClpP body is weakened by the structural changes in the hydrophobic cluster region that further facilitate conformational transitions of BacClpP (Lee et al. 2010). The mutations in the hydrophobic cluster region of BacClpP (including S45C, I19S, L24S, and F49S) resulted in increased ClpP activity only in BacClpP^{S45C}. The other mutants were functionally inactive, as they could only be purified as monomers. Only the BacClpP^{F49S} could be oligomerized into a functional tetradecamer in the presence of ADEP (Lee et al. 2010). Surprisingly, pure LepClpP2^{S40AK41N} could exhibit functional activity and showed increased activity when coupled with LepClpP1. However, both the activities were less compared to LepClpP1P2 (**Figure 4.5**). The oligomerization property of this LepClpP2 mutant also changed, fractionating into heptamer and tetradecamer species (**Figure 4.4**). It would be interesting to investigate such unusual activation of LepClpP as no literature is available citing such mutation (S40AK41N).

The mechanisms of disease pathogenesis in leptospirosis are poorly understood (Murray 2015). The recent advances in molecular genetics, such as the generation of defined mutants, have allowed studying the virulence factors and the biology of *Leptospira* in general (Picardeau 2015). However, pathogenic leptospires are far more difficult to transform than saprophytic leptospires, and further development and improved genetic tools are required (Pappas and Picardeau 2015). Therefore, there will be continued interest in using saprophytic *L. biflexa* as a model bacterium for genetic analyses (Louvel and Picardeau 2007). Knowledge of the

distribution of orthologous genes in *L. biflexa* will be a significant resource for the elucidation of function for genes common to pathogenic and saprophytic strains (Picardeau et al. 2008). Therefore as a proof-of-concept, we attempted to delete one of the *clpP* homologs (LEPBI_I0969) of *L. biflexa*. We aimed to study the significance of *clpP* in the spirochete. It has been reported that genes encoding Clps are highly conserved in both saprophytic and pathogenic strains of *Leptospira* and comprise a part of the core group of genes in the genus *Leptospira* (Picardeau et al. 2008). The two *clpP* homologs in *L. biflexa* exhibited 49-54% identity with its orthologs in pathogenic *L. interrogans* (**Table 4.3**) and further encouraged us to pursue our mutagenesis studies. Though we were able to construct the suicaidal plasmid pMutLept$^{5'\text{-P1P2-pflaBKan-P3P4-3'}}$ (**Figure 4.9**) to knockdown the *clpP2*, we were unsuccessful in generating a *L. biflexa* Δ*clpP2* mutant.

4.5 CONCLUSION

Leptospiral ClpP proteases display a high degree of sequence similarity with various organisms. So, the site-directed mutation at few residues of the crucial hotspots of the LepClpP isoforms was carried out. Preliminary biochemical assays, oligomerization studies, and *in silico* analysis of these LepClpP single/double residue mutant isoforms indicate an unconventional ClpP activation. The gain-of-function of pure LepClpP2^{Y63A} itself is evidence of a diversified functional mechanism of leptospiral ClpP. Further studies in this regard can provide more insights into the nature of leptospiral Clp proteases.

The groundwork for knocking down *clpP2* for generating a *L. biflexa* Δ*clpP2* mutant has been complete. Successful knockdown of *clpP2* in saprophytic *L. biflexa* to study the mutant strain's morphology and growth is further warranted.

4.6 MATERIALS AND METHODS

4.6.1 Multiple sequence alignment of ClpP orthologs and homology modeling of leptospiral ClpP isoforms

Amino acid sequences of ClpP orthologs from different pathogenic bacteria were retrieved from the UniProtKB database (Consortium 2014), and multiple sequence alignment (MSA) was performed using Clustal Omega software (Sievers et al. 2011). MSA is represented using the online tool ESPript (Easy Sequencing in PostScript) for better clarity (Robert and Gouet 2014). The secondary structures used in the study were obtained from the protein data bank (PDB) (Berman et al. 2000). Based on the sequence identities, the tertiary structure models of the ClpP1 and ClpP2 from *L. interrogans* serovar Copenhageni were predicted using the web-based server SWISS-MODEL (Peitsch 1996, Peitsch 1997). *Leptospira* ClpP1 oligomeric

model was developed using the template of the known ClpP1 crystal structure of *C. difficile* (CldClpP1; PDB id: 6mx2), and the ClpP2 model was developed by using the template of the known $ClpP^{A153P}$ crystal structure of *S. pneumonia* ($StrClpP^{A153}$; PDB id: 1y7o). All computational ClpP structures were generated using the program PyMOL.

4.6.2 Site-directed mutagenesis of leptospiral ClpP isoforms

Site-directed single/double amino acid mutation(s) were introduced in the recombinant plasmids pET23a-*clpP1* and pET23a-*clpP2* using Q5 site-directed mutagenesis kit (NEB, catalog no. E0554S). Using the NEBaseChanger tool, primers were designed for single site-directed mutations at 170th Glu to Asp and 172th Asn to Asp residue in rClpP1, resulting in the generation of $LepClpP1^{E170D}$ and $LepClpP1^{N172D}$ variants. The primers were also designed for single site-directed mutation at 63rd Tyr to Ala residue in LepClpP2, resulting in $LepClpP2^{Y63A}$; double site-directed mutations at 40th Ser to Ala and 41st Lys to Asn residues, resulting in $rClpP2^{S40AK41N}$ and; deletions at 126th Ile and 127th Gly residues, resulting in $rClpP2^{I126G127\_del}$ (**Table 4.4**). Sequencing of the generated recombinant pET23a plasmids by outsourcing (Eurofins, India) the plasmid DNA confirmed the mutation in sequences.

Table 4. 4. Primers used in this study for site-directed mutagenesis in the ClpP isoforms of *Leptospira*

Primer Name	Primer sequence (5'-3')	Source or Reference
ClpP1(N172D)F	ACAGAAAGAAgatTTTACATGACAGCAG	This work
ClpP1(N172D)R	ATCTTTTTGAATTTGTTCCAC	This work
ClpP1(E170D)F	GATACAGAAAgatATTTTTACATGACAG	This work
ClpP1(E170D)R	TTTTTGAATTTGTTCCACAG	This work
ClpP2(S40AK41N)F	AGACGAATCTgctaatGATTTAGTTGGTAAAC	This work
ClpP2(S40AK41N)R	GTAACAGGACCCCAGAGA	This work
ClpP2(ΔI126ΔG127)F	GGACAGATTGTAGCACCTG	This work
ClpP2(ΔI126ΔG127)R	ACTAGGTTGATGAATCATCAC	This work
ClpP2(Y63A)F	AATCACTTTTgctATCAATAGTCCCG	This work
ClpP2(Y63A)R	TTTTTACCTGGATCTTTCATTT	This work
F: forward primer. R: reverse primer. The lower case areas indicate the site of mutation		

4.6.3 Expression and purification of LepClpP mutants/variants.

The *Leptospira* ClpP1and rClpP2 variants, except the LepClpP2^{Y63A}, were overexpressed in *E. coli* BL21 (DE3) cells as described for the LepClpP isoforms previously (Dhara et al. 2019). The LepClpP2^{Y63A} expression in the transformed BL21 (DE3) cells were induced with 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). The bacterial cells were cultivated for two hours at 37°C before induction with IPTG. After that, they were induced overnight at 18°C. The cells were then harvested and washed with 1× phosphate buffer saline (PBS pH 7.4, 10 mM sodium phosphate, 137 mM NaCl, and 2.7 mM KCl) by centrifugation at 3000 g for 5 min to remove cellular debris. The LepClpP variants were purified by affinity column chromatography using nickel-nitrilotriacetic acid (Ni-NTA) resins (Invitrogen) as described for the LepClpP isoforms.

4.6.4 Peptide hydrolysis

All pure and mutant LepClpP protein samples were pre-incubated for 24 at 4°C before every peptidase and protease assays.

Peptidase activity was measured by the production rate of fluorescent AMC (7-amino-4-methyl coumarin) after cleavage from fluorogenic peptide substrates, as described previously for the LepClpP isoforms. The peptide substrate used in the hydrolysis assay was N-succinyl-Leu-Tyr-AMC (Suc-LY-AMC) (substrate S1) (Sigma). Peptidase assays were performed in black flat-bottom 96-well plates (Invitrogen) at 37°C. Each well in the black plates contained fluorogenic peptide substrate (0.1 mM) and LepClpP mutant isoforms (0.0125 μg μL^{-1}) in 80 μL of ClpP peptidase activity buffer (50 mM phosphate buffer pH 7.6, 100 mM KCl, 5% glycerol). Fluorescence was measured in the Infinite M200Pro plate reader (Tecan) at 380 and 460 nm wavelength of excitation and emission, respectively.

4.6.5 Protease assay of leptospiral ClpP variant –ClpP2^{Y63A}.

Pure LepClpP isoforms, their mixture, LepClpP1P2^{Y63A} and LepClpP2^{Y63A} (1 μg each) were pre-incubated for 24 h at 4°C in ClpP protease activity buffer (50 mM Tris-Cl pH 7.0, 50 mM KCl, 1 mM DTT, 8 mM $MgCl_2$, 5% glycerol). Each of the pre-incubated protein samples was then mixed with 10 μM of fluorgenic FITC-casein substrate in separate tubes in a total reaction of 50 μL. The reaction in the tubes was continued in the dark for 2 h at 37°C. After 2 h, the reactions were terminated with trichloroacetic acid (TCA). Hydrolysis of the FITC-casein was monitored via i-TECAN Infinite M200 plate reader (excitation: 492 nm; emission: 519 nm) in a 96-well black plate (Invitrogen). In an alternative assay format, bovine β-casein was used as a substrate to evaluate the protease activity of LepClpP2^{Y63A}. One microgram of LepClpP1P2,

LepClpP1P2^{Y63A}, and LepClpP2^{Y63A} was incubated for 24 h at 4°C in ClpP protease activity buffer. Each of the incubated protein samples was separately mixed with 10 µM β-casein in a total reaction of 50 µL. The reactions were continued for 2 h at 37°C. From the total reaction volume, a small volume (20 µL) of the reaction was terminated at the 0th and 2nd hour after adding sample buffer and heating for 10 min at 95°C. The reaction products of each time point were resolved on 12% SDS-PAGE and visualized by Coomassie staining.

4.6.6 Native Polyacrylamide Gel Electrophoresis.

Pure LepClpP mutant isoforms in 50 mM Tris-Cl pH 8.0, 100 mM NaCl, and 10% glycerol were incubated 24 h at 4°C. The incubated samples were mixed with 3x native sample buffer (240 mM Tris-HCl pH 6.8, 30% glycerol, 0.03% bromophenol blue**)** (Preissler et al. 2015). The LepClpP mutant isoform subunits interaction in solution was analyzed on a 7.5% native gel resolving for 2 h at 120 V. The resolved proteins were visualized using Coomassie stain and compared with known molecular weight standard protein markers (Invitrogen, cata1og no.928387).

4.6.7 Leptospiral strains and culture conditions.

The saprophytic *L. biflexa* serovar Patoc strain Patoc1 was used in this study. *Leptospira* organisms were cultivated in the commercially available liquid Ellinghausen-McCullogh-Johnson-Harris (EMJH) medium.

4.6.8 Generation of *L. biflexa clpP2* deletion mutant.

A pET23a plasmid containing an ampicillin marker (AmpR) has been used to generate the suicidal plasmid for deleting the *clpP2* gene (*LEPBI_0969*) in *L. biflexa* serovar Patoc strain Patoc1. The empty suicidal plasmid construct was developed previously by another member in our laboratory. Briefly, the suicidal plasmid (pMutLept) contains a kanamycin-resistant cassette (KanR) driven by the promoter of the *flaB* gene from *L. interrogans* and flanked by two multiple cloning sites. For the allelic exchange to generate the Δ*clpP2* mutant from wild-type *L. biflexa* strain Patoc1, two separate PCRs were performed. One to amplify a 1000-bp 5' homologous arm (5'arm/P1P2) that was located upstream of the *clpP2* gene and another to amplify a 1000-bp 3' homologous arm (3'arm/P3P4) that was located downstream of *clpP2* gene using the primers p1p2-F/p1p2-R and p3p4-F/p3p4-R (**Table 4.5**). The pMutLept was digested with *Hind*III and *Sal*I endonucleases (NEB) to insert the P1P2 flanking region at multiple cloning site- I (MCS- I) and *Nhe*I and *Bgl*II to insert the P3P4 flanking region at MCS-II to form a 5'-P1P2-pflaBKan-P3P4-3' segment under the action of T4 DNA ligase. The Amp^r of the suicidal plasmid construct pMutLept$^{5'\text{-P1P2-pflaBKan-P3P4-3'}}$ was inactivated before electro-

transformation. The competent wild-type *L. biflexa* strain Patoc 1 was prepared and electrotransformed, as described previously (Girons et al. 2000). Briefly, 50 mL of a log-phase culture of *L. biflexa* strain Patoc 1 (2×10^8 to 5×10^8 cells mL^{-1}) were pelleted at 4,000 × g for 20 min at 20°C, washed twice with a half volume of sterile, deionized water (for an injectable preparation, pH 6), resuspended with 200 µl of water (final volume, 300 µL), and used immediately. 50 µL of cell suspension (4×10^9 cells) was mixed with 1 to 2 µg of plasmid DNA (in 5 µL of water), kept for 10 min at 4°C, and then transferred to a 0.2-cm-diameter ice-cold electroporation cuvette at 4°C. One pulse was delivered from a gene pulser with a Pulse controller (set at 1.8 kV, 25 µF, and 200 Ω, producing a time constant of 4.5 ms; Bio-Rad). One milliliter of EMJH liquid medium was immediately added to the cuvette, and the cells were transferred to a 15-ml culture tube. Cultures were incubated at 28-30°C for 24 to 48 h with shaking in the absence of selection. Then 500 µL of leptospires were inoculated in solid EMJH agar plates with 50 µg mL^{-1} kanamycin (Sigma). Plates were incubated for 4 to 6 days at 30°C. The colonies obtained were inoculated in a kanamycin-containing EMJH medium for incubation at 28-30°C to obtain the Δ*clpP2* mutant.

Table 4. 5. Primers used in this study for mutagenesis plasmid construct

Primer name	Sequence (5'-3')
p1p2-F(HindIII)	AAGCTTACCAAAGTTAAAGAAGATCAAAATCC
p1p2-R(SalI)	GTCGACAGTTTACTCTTCTTTCCCGCTA
p3p4-F(NheI)	GCTAGCAAGGGGGCAACTCTCCATG
p3p4-R(BglII)	AGATCTTTTTAAACTTTCGATCGTGAGTTCA
LEPBI_ I0969_F	ATGCCAGAAGAAGAAACACCA
LEPBI_ I0969_R	TTAGACGACGTCAATTGTGTTTAC
pMutLept-Amp_F	TTACATTTACCAATGCTTAATCA
pMutLept-Amp_R	AGTATTCAACATTTCCGTG
F: forward primer. R: reverse primer. The italicized areas indicate the sites of endonucleases	

4.7 REFERENCES

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Chapter 5

5.1 CONCLUSION

This body of work biochemically and functionally describes the ClpP system in *L. interrogans.* Our main aim in the project was to demonstrate the biological significance of targeting ClpP in order to introduce ClpP as a new target against leptospirosis. To date, not much is known about the Clp protease-chaperone system in *Leptospira* except LepClpB (Lourdault et al. 2011, Krajewska et al. 2016, Krajewska et al. 2017) and LepHslUV (LepClpYQ) (Dong et al. 2017). The *L. interrrogans* ATPase chaperone ClpB and the ATPase-dependent Clp protease HslUV were found to be related to bacterial virulence. The deletion of these genes in the organism ushered in a failure of the bacteria's survival in the hosts and transmission of leptospirosis (Dong et al. 2017, Lourdault et al. 2011). The core ClpY in *Leptospira* is a threonine protease, but to date, no work is reported about the other core ClpP, a serine protease. ClpP in prokaryotes is the main cytosolic house-keeping protease that takes care of the unfolded or misfolded proteins (Alexopoulos et al. 2012, Laederach et al. 2014, Hall et al. 2016). Thus we set out to understand the operative *Leptospiral* ClpP system. This included how each ClpP isoform assembled, how they behaved *in vitro,* and the possible structure of the ClpP tetradecamer predicted by the computational approach. We accomplished this initial goal by biochemically characterizing the *Leptospiral* ClpP system.

We establish that the operative *L. interrogans* LepClpP protease is an unusual core ClpP particle composed of both isoforms LepClpP1 and LepClpP2. Regarding genetic organization and enzyme function, *Leptospiral* Clp protease appears very identical to the *Chlamydia clpP* gene arrangement (Pan et al. 2019) among the characterized Clp systems in bacteria (**Figure 5.1**). Two distinct LepClpP homologs from different and distant genetic loci have to assemble to form a functionally active protease. It may be hypothesized that such a functional connection of distant genetic loci with regulated proteolysis could play a decisive role in the biology of *Leptospira*. The functional activation mechanism would ensure that the LepClp protease activity is only engaged in efficient replication and transcription of both genomic regions, thus representing a safeguard mechanism of LepClpP activity on the genomic level. The study also confirms that the assembly of physiological chaperone LepClpX and LepClpP1P2 protease is a prerequisite of the protein degradation process in an energy-dependent manner.

	M. tuberculosis	*C. diffcile*	*L. monocytogenes*	*P. aeruginosa*	*C. trachomatis*	*L. interrogans*
clpP1/clpP2 organization	***Single operon***	***Distinct operons***	***Distinct operons***	***Distinct operons***	***Distinct operons***	***Distinct operons***
ClpP1	 14-mer inactive	 14-mer active	 7-mer inactive	 14-mer active	 7-mer inactive	 21-mer inactive
ClpP2	 14-mer inactive	 14-mer active	 14-mer active	 7-mer inactive	 7-mer inactive	 14-mer inactive
ClpP1P2	 14-mer active	**n.d.**	 14-mer active	**n.d.**	 14-mer active	 14-mer active

Figure 5. 1. Overview of Clp protease systems from bacterial species that encode two ClpP homologs.

The functional Clp protease complex of *L. interrogans* is unique among the previously identified Clp systems from other bacteria regarding its genetic organization in combination with its assembled structure and activity *in vitro*. In *M. tuberculosis*, MycClpP1 and MycClpP2, encoded by a single bicistronic operon, may either form inactive homo 14-mers or functionally active hetero14-mers using the non-natural activator peptide Z-Leu-Leu20 (regarded as active*)(Akopian et al. 2012). ClpP homologs from either *C. difficile*, *L. monocytogenes*, or *P. aeruginosa*, which are encoded in distinct operons, may form either functionally active homo 14-mers as well as hetero 14-mers (Lavey et al. 2018, Dahmen et al. 2015, Krajewska et al. 2017). Thus, in each of these situations, an active ClpP complex can result from the same operon or even a single ClpP homolog. Only chlamydial ClpP homologs are encoded by distinct operons at different locations of the chlamydial genome like LepClpP homologs, and they do not form active homo 14-mers, but inactive homo 7-mers (Pan et al. 2019) . But the Lep ClpP homologs forms the inactive homo- 21-mer or 14-mer. Only the combination of the two homologs from different operons results in the assembly of an active hetero 14-mer, which may resemble a safeguard mechanism of ClpP activity on the genomic level.

Besides general proteolysis, the Clp system is also involved in the degradation of regulatory proteins such as transcriptional regulators (Barik et al. 2010, Dziedzic et al. 2010, Raju et al. 2014). The essential nature and multi-component assembly make it a promising drug target (Moreno-Cinos et al. 2019). Indeed several compounds are active on the Clp system, illustrating that its deregulation is detrimental to the bacterial cell. One such compound is natural antibiotic ADEP1 that generally acts through overstimulation of the ClpP protease (Gersch et al. 2015, Li et al. 2010) and adversely affects the morphology of microorganisms by degrading cell division proteins (Sass et al. 2011). Thus, this study investigated the detrimental effect of ADEP1 on binding to its target ClpP in *L. interrogans* grown under *in vitro* conditions. We demonstrated that ADEP1 dependent chemoactivation of LepClpP1P2 is a time-dependent process. The outcomes indicate that apart from the regular interaction sites of ADEP1 in the apical surface of ClpP1P2 tetradecamer, ADEP1 also interacts with the Ser98 residues of LepClpP1 to produce enhanced proteolytic activity. The suggestive model of chemoactivation of ADEP-bound LepClpP1P2 is shown in **Figure 5.2**. These results lay the groundwork for pursuing the biological significance of the Clp system in *L. interrogans* serovar Copenhageni.

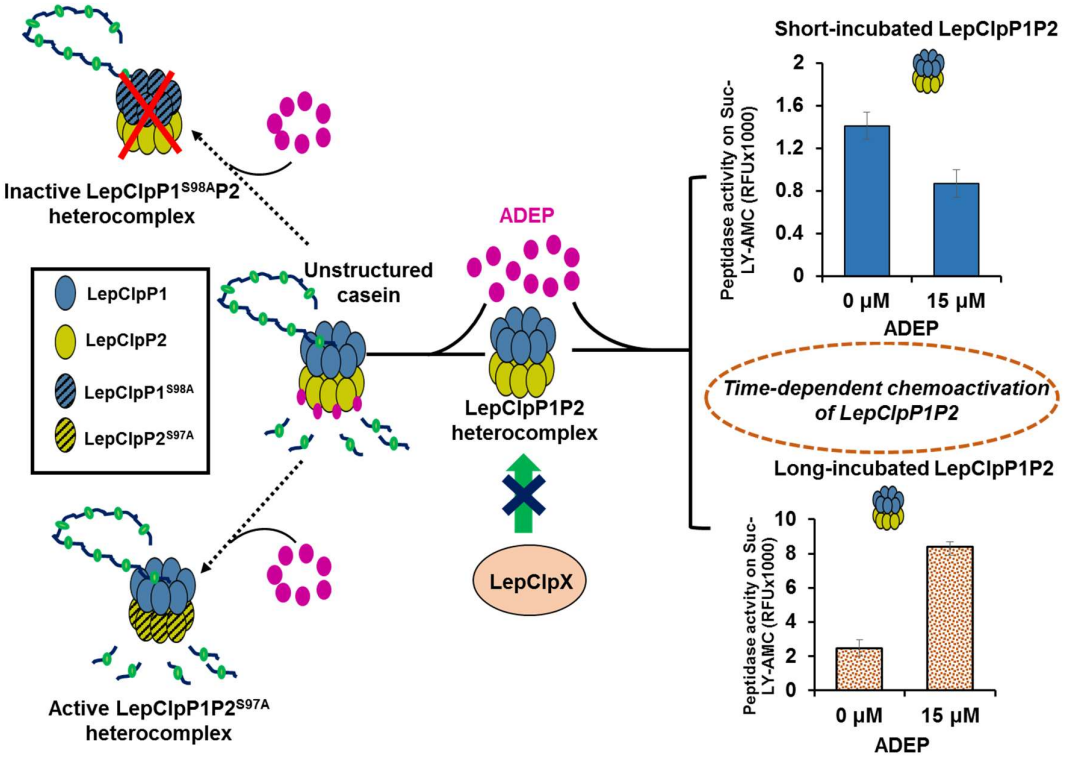

Figure 5. 2. Protease/Peptidase activity of LepClpP1P2 heterocomplex and their mutants in the presence of ADEP.
(Left panel) The ADEP-bound LepClpP1P2 heterocomplex can switch to an unregulated protease and degrades the unstructured casein substrate autonomous of the cognate chaperone LepClpX. The site-directed mutagenesis of a catalytic serine residue in either of the ClpP isoforms proposes a second interaction site for the ADEP, where LepClpP1^{S98A}P2 is inactive (denoted by a red cross) and LepClpP1P2^{S97A} is active in the presence of ADEP. **(Right panel)** The peptidase activity of the ADEP-bound LepClpP1P2 heterocomplex on the fluorogenic dipeptide substrate is conditional on the duration of the self-compartmentalization of each of the ClpP isoforms. The short-incubated self-assembled LepClpP1P2 revealed inhibition in the peptidase activity in the presence of the ADEP while the LepClpP1P2 (long-incubated) activity became enhanced.

There are several advantages of targeting ClpP: (l) Both inhibition and activation are possible; each strategy can affect different aspects of bacterial pathogenicity (Gersch et al. 2014, Brotz-Oesterhelt et al. 2005). It allows evaluating the therapeutic potential of two strategies on a single target-a rare phenomenon in antibacterial drug discovery. (2) The diverse regulatory roles of ClpP (e.g., growth, motility, virulence, stress response, sporulation), disruption of its natural activity would have pleiotropic effects that compensatory mutations may not easily resolve (Bhandari et al. 2018) (3) ClpP activation demonstrates efficacy against both actively growing and dormant persister cells (Conlon et al. 2013), a necessity for improved antibacterial treatments. (4) ClpP is essential for pathogenicity but not survival (except *M. tuberculosis*) in most studied organisms (Bhandari et al. 2018). Thus, inhibiting may negatively affect the virulence of pathogens but preserve the viability of beneficial microbes. (5) Targeting non-

essential virulence regulators like ClpP is likely to impair the organism's infectivity without imparting typical selective pressures that drive resistance (Lavey et al. 2018).

Despite the advancements in ClpP research, major questions regarding the clinical potential of targeting this protease persist. Although the general involvement of ClpP in bacterial virulence is well-established, the distinct functional attributes regulated by ClpP are organism-dependent. Moreover, while resistance to ADEPs has been generated in laboratory settings (*E. coli, B. subtilis, E. faecalis, S. pneumoniae, S. aureus*), all studies have been conducted on single clpP containing organisms. While ClpPs from single isoform expressing bacteria has been studied extensively, understanding the function and regulation of systems with more than one clpP gene are far more limited (Lavey et al. 2018). Interrogating ClpP systems' behavior in multi-isoform containing organisms is expected to reveal additional insight into mechanisms of resistance development that should be considered, both during the validation of a target and in its clinical exploitation. In these contexts, it will be interesting to elucidate the behavior, biological relevance, and therapeutic potential of the ClpP system in pathogenic *L. interrogans*. There is a considerable amount of work that can be pursued in the near future to explore the *L. interrogans* caseinolytic proteases. In our study, a major portion was focused on the *in vitro* characterization of *Leptospiral* ClpPs. It would be interesting to further analyze the behaviour of various *clp* genes under environmental stress conditions for understanding their importance in the survival of the spirochetes. The continuation of the gene knockdown study will further establish the necessity of *clp* genes in *Leptospira* as reported in other microorganisms.

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Appendix A: Supporting data for Insights to the assembly of functionally active leptospiral ClpP1P2 protease complex along with its ATPase chaperone ClpX

Table A. 1. Oligonucleotides used for quantification of *Leptospira* transcripts

Primer	Sequence (5'-3')
qclpP1_F	ATGGAGCGTAATCCCGTATGTG
qclpP1_R	AAACAACAATTGAGCTGTAATGACA
qclpP2_F	ATGCCAGAAACAGAGAAAAAATCG
qclpP2_R	CCGGGACTATTGATATAAAAAGTGAT
16S rRNA_F	TTATTGCTCGGAGATGAGCC
16S rRNA_R	TTCAGGGTTCCCCCCATT

Table A. 2. Dynamic light scattering (DLS) data of *Leptospira* ClpP isoforms (LepClpP)

Sample Name	Polydispersity (nm)	Pd Index	Count rate per second (cps)	Estimated Molecular Weight (kDa)* (Mean ± SD) *[Major peak]
LepClpP1_1 h or 24 h incubation	18.1	0.498	3,44,000	657.6±198.9
LepClpP2_1 h or 24 h incubation	7.3	0.219	1,82,000	474.1±172.4
LepClpP isoforms mixture_1 h incubation	21.3	0.570	2,66,900	658.1±182.5
LepClpP isoforms mixture_24 h incubation	9.4	0.257	2,01,200	591.0±179.8

Pd –Polydispersity; cps – count per second

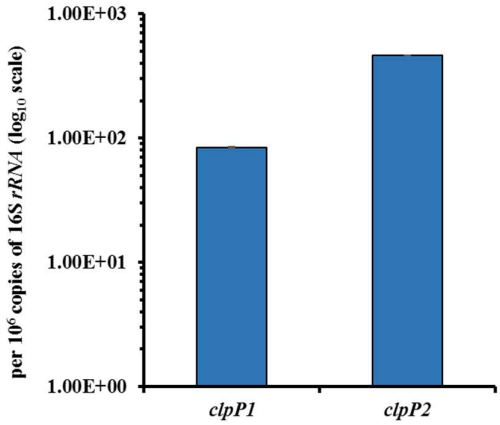

Figure A. 1 Differential transcription analysis of *Leptospira clpP1* and *clpP2* under *in vitro* condition.
Transcript analysis of *clpP1* and *clpP2* genes by qRT-PCR of cDNA synthesized from *Leptospira interrogans* serovar Copenhageni grown in EMJH (Ellinghausen-McCullough-Johnson-Harris) medium. The gene transcription was calculated based on threshold cycle (C_T) values by the use of $2^{-\Delta\Delta CT}$ method and normalized against 16S rRNA values. Error bars represent the standard deviations (SDs) from two independent qRT-PCR analyses.

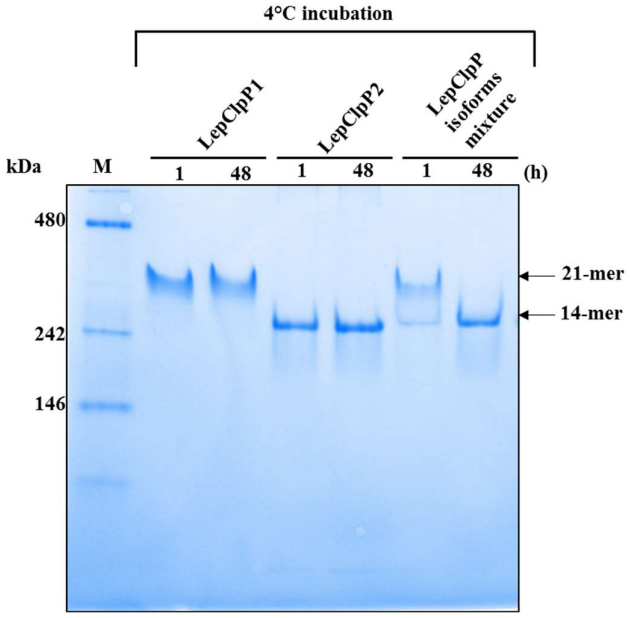

Figure A. 2 Oligomerization of pure LepClpP isoforms and their mixture.
Pure LepClpP isoforms (1.0 µg each) and its mixture (0.5 µg of each of the pure LepClpP isoforms) were incubated for 1 h and 48 h at 4°C before analysing the samples on native-PAGE. The samples were resolved on 7.5% native gel and visualized by Coomassie staining.

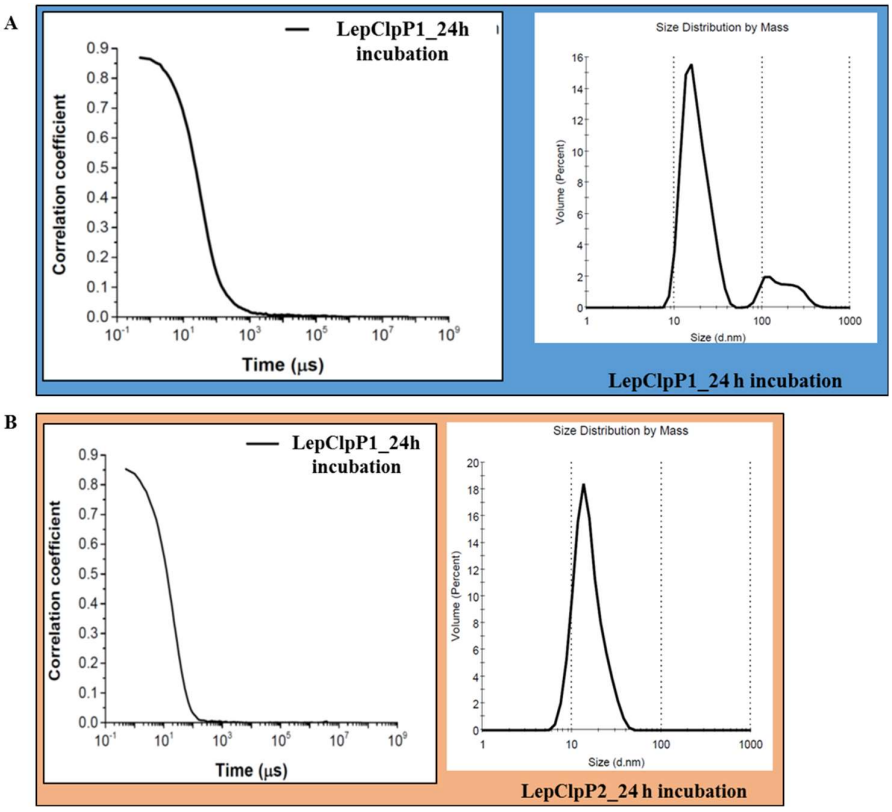

Figure A. 3 DLS analysis of LepClpP isoforms.
(A and B) Correlation coefficient and mass distribution graphs of LepClpP isoforms after 24 h incubation (4°C).

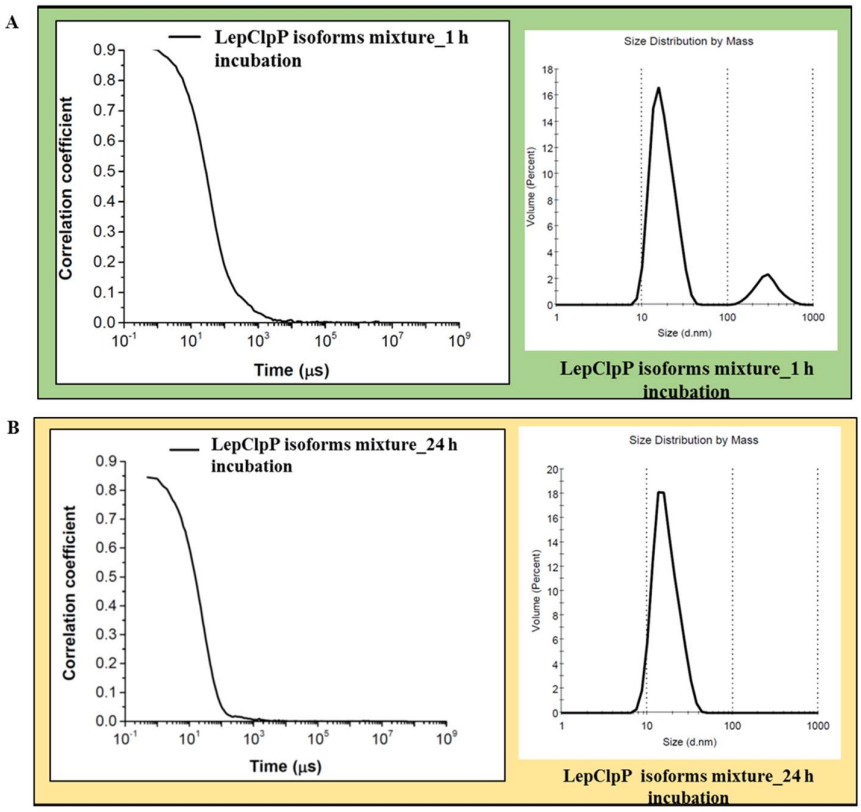

Figure A. 4 DLS analysis of LepClpP isoforms mixture.
(A) Correlation coefficient and mass distribution graphs of LepClpP isoforms mixture after 1 h incubation (4°C) **(B)** Correlation coefficient and mass distribution graphs of LepClpP isoforms mixture after 24 h incubation (4°C).

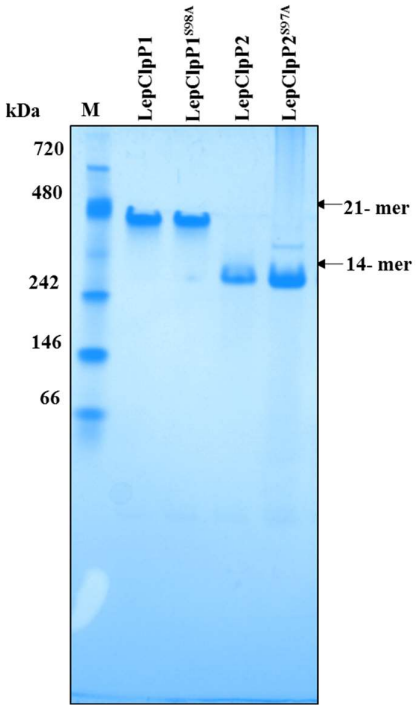

Figure A. 5. Oligomerization of LepClpP serine mutants.
Native 4-20% gradient gel image of pure LepClpP serine mutants.

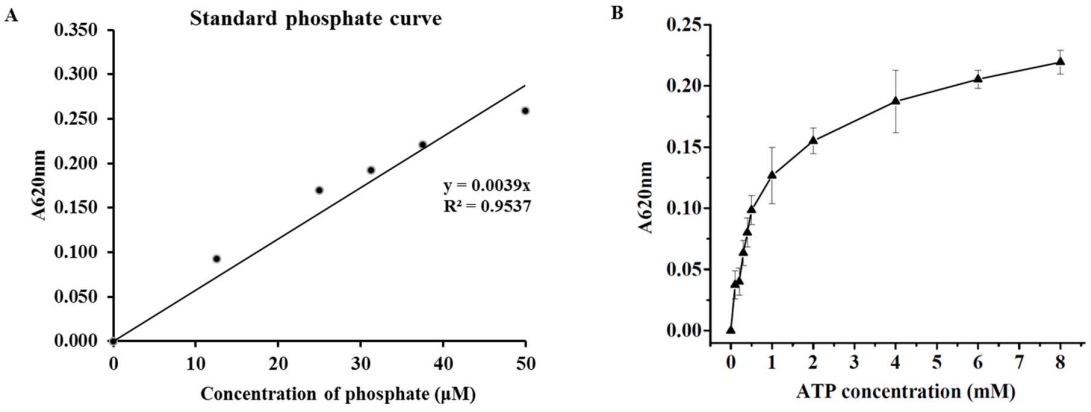

Figure A. 6. Estimation of LepClpX ATPase activity.
(A) Phosphate standard curve generated using known standard phosphate concentrations provided in ATPase assay kit. **(B)** Absorbance readings at 620 nm of LepClpX ATPase activity assay. Experiments were performed twice independently in duplicate and error bars represent the SDs from the two independent experiments.

Appendix B: Supporting data of ADEP1 activated ClpP1P2 of *Leptospira*, an ideal Achilles' heel to deregulate proteostasis and hamper cell survival

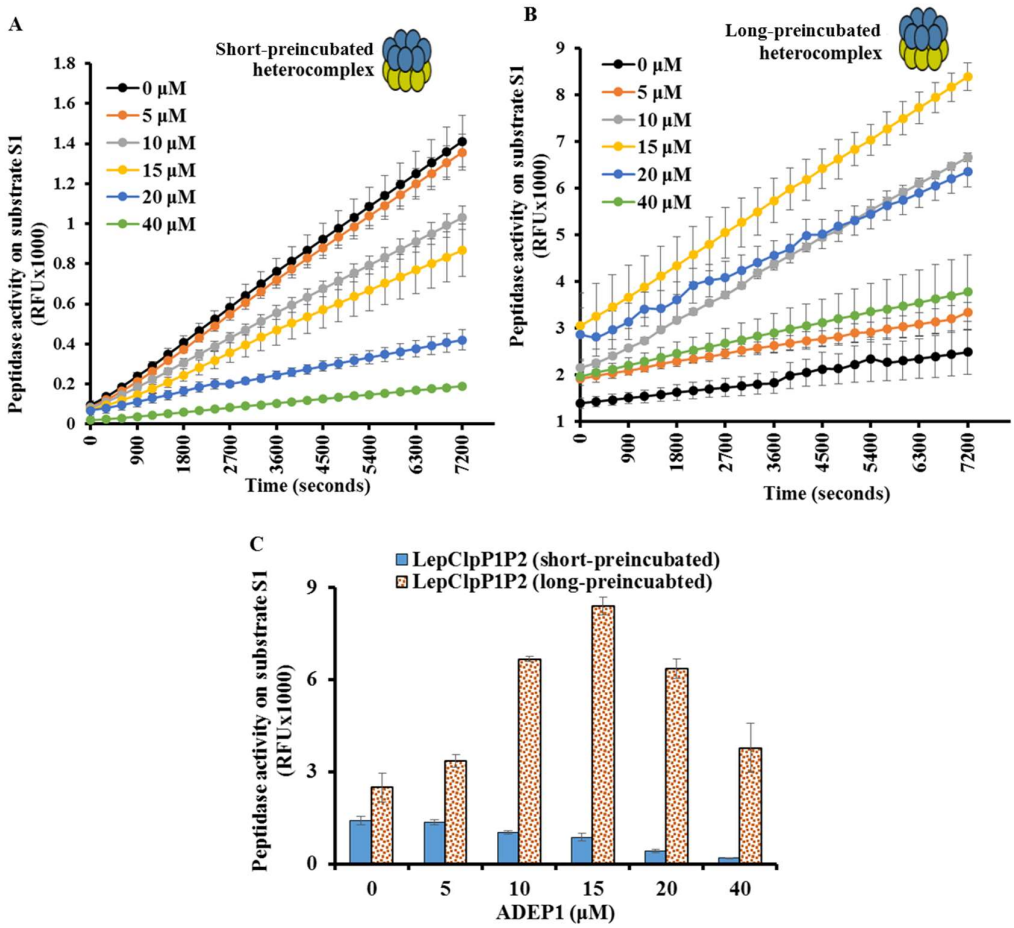

Figure B. 1. Absolute peptidase activity of short-preincubated LepClpP1P2 in the presence of different ADEP1 concentration is lower than long-preincubated LepClpP1P2.
(A) and **(B)** Peptidase activity of short- and long-preincubated LepClpP1P2 on the dipeptide substrate S1 in the presence of a variable amount of ADEP1. Peptide degradation was measured fluorometrically as a relative fluorescent unit (RFU×1000) at an interval of 5 min for 2 h. **(C)** Peptidase activity of short- and long-preincubated LepClpP1P2 on the dipeptide substrateS1, where end-point fluorescence was measured after 2 h of the enzymatic reaction. The error bars indicate the respective standard errors of the mean (SEM) from the two independent experiments performed.

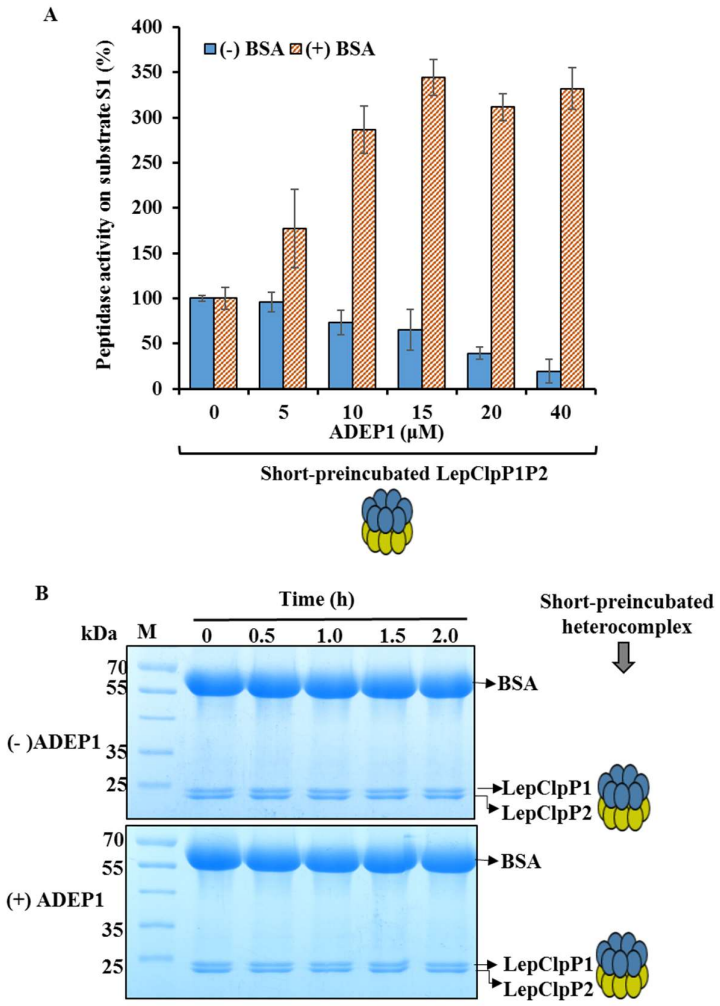

Figure B. 2. Peptidase activity of ADEP1-bound LepClpP1P2 (short-preincubated) gets enhanced on BSA's addition but the protease activity on BSA was not discernible.
(A) Comparison of peptidase activity of rClpP1P2 stimulated by ADEP1 in the presence (+) or absence (-) of bovine serum albumin (BSA). Peptidase activity of LepClpP1P2 is represented as a percentage (%), wherein the end-point fluorescence was measured after 2 h of the enzymatic reaction. The measured end-point fluorescence value of the LepClpP1P2 (containing no ADEP1) as control was considered 100% for measuring the relative peptidase activity. ADEP1 increases the peptidase activity (~3.4-fold) of rClpP1P2 in the (+) of BSA. For clarity, the cartoon representation of ClpP tetradecamer used is presented. The error bars indicate the respective standard errors of the mean (SEM) from the two independent experiments performed. **(B)** Denaturing gel electrophoresis showing the activity of LepClpP1P2 (short-preincubated) on the BSA in the presence (+) or absence (-) of ADEP1. ADEP1 does not stimulate rClpP1P2 (represented in a red cross) for the degradation of BSA but its presence can enhance the peptidase activity of ADEP-bound LepClpP1P2.

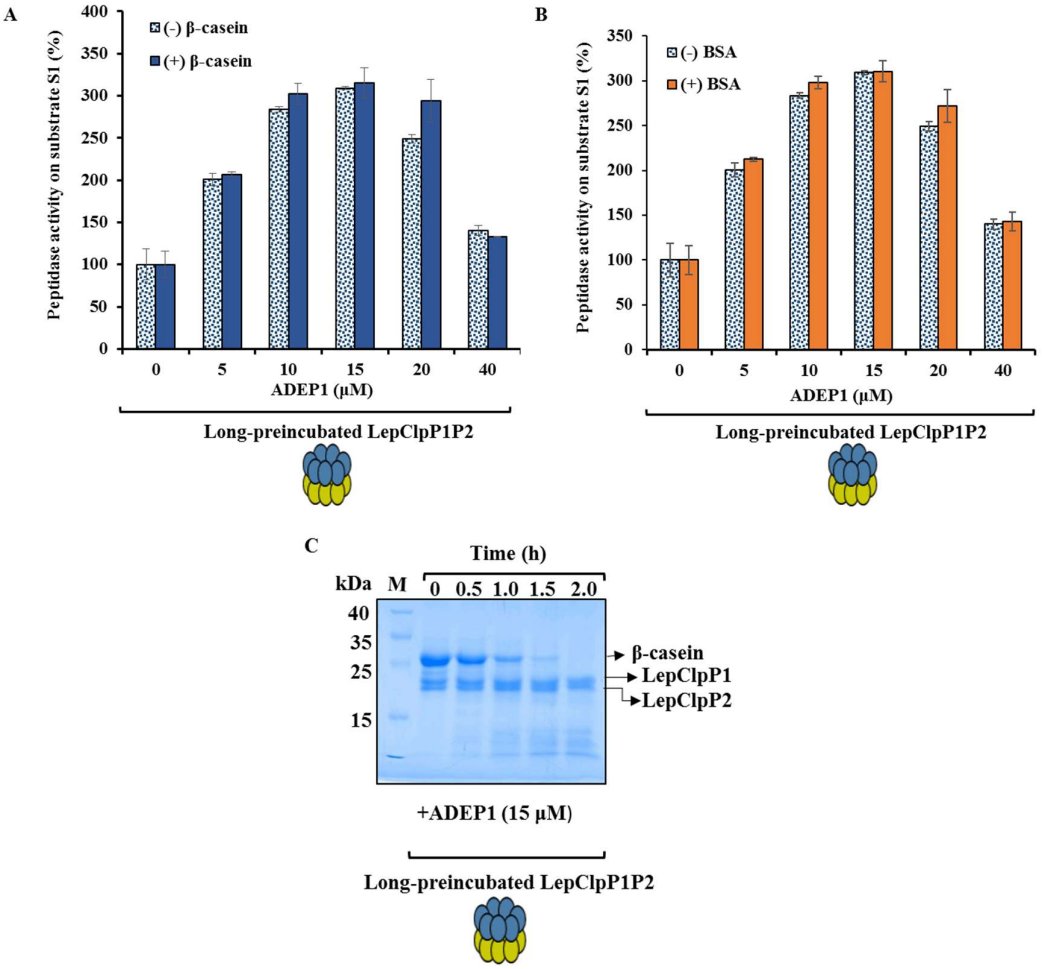

Figure B. 3. The peptidase activity of LepClpP1P2 (long-preincubated) bound to ADEP1 demonstrated a gain of activity while supplementation of β-casein or BSA did not lead to any additional stimulation in its activity.

(A and B) Comparison of peptidase activity of LepClpP1P2 stimulated by ADEP1 in the presence (+) or absence (-) of β-casein/BSA. Peptidase activity of rClpP1P2 is represented as a percentage (%), wherein the end-point fluorescence was measured after 2 h of the enzymatic reaction. The measured end-point fluorescence value of the LepClpP1P2 (containing no ADEP1) as control was considered 100% for measuring the relative peptidase activity. The error bars indicate the respective standard errors of the mean (SEM) from the two independent experiments performed. (C) Denaturing gel electrophoresis showing the reaction products (0-2 h) after stimulation of peptidase activity of LepClpP1P2 (long-preincubated) in the presence 15 μM of ADEP1 and supplementation of β-casein. The LepClpP1P2 (long-preincubated) can degrade the unstructured model substrate β-casein with the abolition of its ClpP subunits' self-cleavage.

List of Publications

National and International Conferences

1. **Anusua Dhara** and Manish Kumar (2019). "Potential application of antibiotic acyldepsipeptide (ADEP1) for targeting ClpP protease of pathogenic *Leptospira*." Global Bio-India Conference at Aero-city, New Delhi.
2. **Anusua Dhara** and Manish Kumar (2018). "Two isoforms of Caseinolytic proteases (ClpPs) of *Leptospira interrogans* are essential for functional activity." 1st World Congress on Infectious Diseases & Antibiotics at IISc, Bangalore.
3. **Anusua Dhara** and Manish Kumar (2016). "Cloning, expression, and purification of Caseinolytic proteases of pathogenic *Leptospira interrogans* Copenhageni Strain Fiocruz L1-1302016." 57th Annual Conference of Association of Microbiologists of India and International Symposium on "Microbes and Biosphere" at Gauhati University, Assam.
4. **Anusua Dhara,** Yogesh Baid, Aman Prakash, and Manish Kumar (2016). "Leptospirosis- An underrated disease." Research Conclave, IIT Guwahati.

International Journals

1. **Dhara, A.**, Hussain, M. S., Kanaujia, S. P., & Kumar, M. (2021). Acyldepsipeptide activated ClpP1P2 macromolecule of Leptospira, an ideal Achilles' heel to hamper the cell survival and deregulate ClpP proteolytic activity. *Research in Microbiology*, 103797. doi: 10.1016/j.resmic.2021.103797
2. Choudhury, M., **Dhara, A.** and Kumar, M. (2021) 'Trigger Factor in Association with the ClpP1P2 Heterocomplex of Leptospira Promotes Protease/Peptidase Activity', *ACS Omega, 6*(2), 1400-1409. doi: 10.1021/acsomega.0c05057
3. Ghosh, K. K., Prakash, A., **Dhara, A.**, Hussain, M. S., Shrivastav, P., Kumar, P., Balamurugan, V., & Kumar, M. (2019). Role of supramolecule ErpY-like lipoprotein of Leptospira in thrombin-catalyzed fibrin clot inhibition and binding to complement factors H and I, and its diagnostic potential. *Infection and Immunity, 87*(12). doi: 10.1128/IAI.00536-19

4. **Dhara, A.**, Hussain, M. S., Datta, D., & Kumar, M. (2019). "Insights to the Assembly of a Functionally Active Leptospiral ClpP1P2 Protease Complex along with Its ATPase Chaperone ClpX." *ACS Omega, 4*(7), 12880-12895. doi: 10.1021/acsomega.9b00399

Conferences/Workshops attended

1. 9th TCS Annual Event and Flow Cytometry Workshop on "Flow Application in Basic, Applied and Clinical Biology" at IIT Guwahati, Assam (2016).