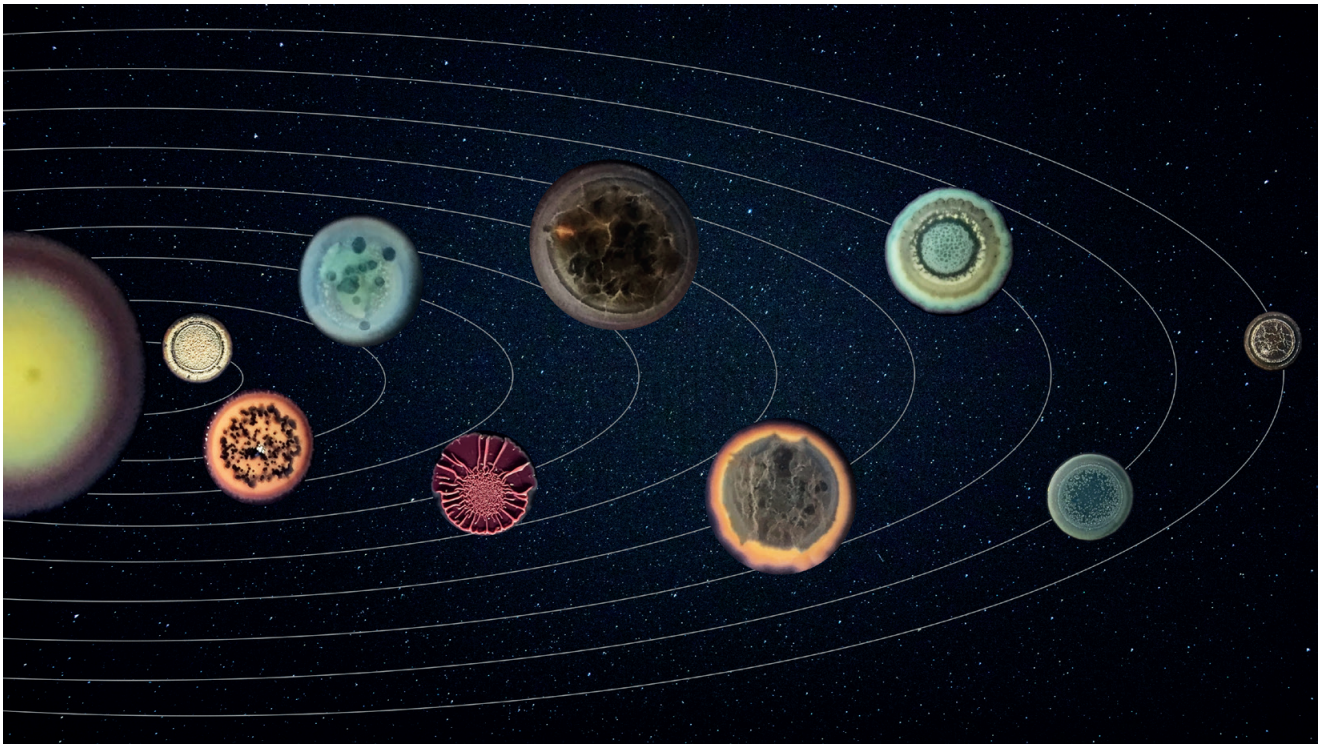
Thesis for doctoral degree (Ph.D.)
2020

FtsH protease and ClpG disaggregase confer fitness advantages to the worldwide prevalent *Pseudomonas aeruginosa* clone C



Shady Mansour Kamal

Karolinska Institutet

Department of Microbiology, Tumor and Cell Biology
Karolinska Institutet, Stockholm, Sweden

FTSH PROTEASE AND CLPG DISAGGREGASE CONFER FITNESS ADVANTAGES TO THE WORLDWIDE PREVALENT *PSEUDOMONAS AERUGINOSA* CLONE C

Shady Mansour Kamal

Karolinska Institutet

Stockholm 2020

Cover picture: *''Pseudomonas Universe''*
The picture shows colonies of *Pseudomonas aeruginosa* Clone C and non-clone C strains of clinical and environmental origins arranged as the planets of our solar system. The colonies were grown on Congo Red agar plates at room temperature. The full results are published in Kamal *et al.,* 2019. The cosmic background photo is by Paul Volkomer from Unsplash.

Published by Karolinska Institutet.
Printed by Eprint AB 2020

ISBN 978-91-7831-853-7

FtsH protease and ClpG disaggregase confer fitness advantages to the worldwide prevalent *Pseudomonas aeruginosa* clone C

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Publicly defended on Friday 12th June 2020, 09.00

By

Shady Mansour Kamal

Principal Supervisor:
Professor Ute Römling, PhD
Karolinska Institutet
Department of Microbiology,
Tumor and Cell Biology

Co-supervisor(s):
Changhan Lee, PhD
University of Michigan
Department of Molecular,
Cellular & Developmental Biology

Professor Arne Holmgren, PhD
Karolinska Institutet
Department of Medical
Biochemistry and Biophysics

Opponent:
Professor Franz Narberhaus, PhD
Ruhr Universität Bochum
Department of Biology and Biotechnology

Examination Board:
Professor Anna Norrby-Teglund, PhD
Karolinska Institutet
Department of Medicine

Professor Christiane Funk, PhD
Umeå University
Department of Chemistry

Associate Professor Herwig Schüler, PhD
Karolinska Institutet
Department of Biosciences and Nutrition

In loving memory of my sister Haidy

To my parents

ABSTRACT

Pseudomonas aeruginosa is an environmental bacterium and a frequent nosocomial pathogen causing a wide range of opportunistic infections, especially in immunocompromised patients. Clone C is one of the most prevalent groups of closely related strains distributed worldwide in the environment, such as in natural aquatic habitats, and the clinical settings, such as in patients with an underlying functional impairment of the cystic fibrosis transmembrane conductance regulator. Clone C strains specifically harbor the horizontally-transferred genomic island PACGI-1. One border of the genomic island contains the transmissible locus of protein quality control (TLPQC), alternatively called the Locus of Heat Resistance (LHR) in other bacteria, predominantly encoding stress-related gene products such as proteins involved in proteostasis. Among those gene products is a xenolog of the gene encoding the AAA+ (ATPase associated with diverse cellular activities) membrane-bound protease FtsH termed FtsH2 and the AAA+ disaggregase ClpG termed $ClpG_{GI}$. In clone C isolates, *ftsH2* and $clpG_{GI}$ encoded on TLPQC exist in addition to the core genome homologs *ftsH1* and *clpG*. This thesis investigates the divergent and convergent roles of the genomic island and core genome copies of *ftsH* and *clpG* in fitness and prevalence of the aquatic clone C isolate *P. aeruginosa* SG17M.

Paper I identifies *ftsH1* as a pleiotropic gene in *P. aeruginosa* SG17M, affecting a multitude of phenotypes related to fitness and adaptation such as growth, motility, biofilm formation, antibiotic resistance, autolysis, secondary metabolite secretion and oxidative and heat shock stress. In the absence of *ftsH1*, the TLPQC locus copy *ftsH2* backs up *ftsH1* functionality. FtsH1 and FtsH2 share highly conserved functional AAA+ ATPase and protease domains and form homo- and hetero-oligomers with FtsH2 distinctively produced in the late stationary phase. However, mainly FtsH1 controls the levels of the heat-shock transcription factor RpoH (σ^{32}). Using FtsH trap variants in an *in vivo* crosslinking/*in vitro* pull-down experiment shows that the phenazine biosynthesis protein PhzC is a novel substrate for FtsH1 in *P. aeruginosa* SG17M.

Paper II investigates the molecular basis of the differential *in vivo* functionality of FtsH1 and FtsH2 in *P. aeruginosa* SG17M. We show that the N-terminal 151 amino acids of FtsH1 are required for FtsH1 functionality and, consequently, optimal growth of *P. aeruginosa* SG17M. The periplasmic domain and the short glycine-rich cytoplasmic linker connecting the N-terminus to the AAA+ module are particularly crucial for the optimal functionality of FtsH1. Moreover, *in vitro* biochemical analysis of the purified FtsH proteases shows that FtsH1 and FtsH2 are homo-hexamers and active ATPases with differential degradation activity towards model substrates such as FITC-casein and Arc-st11-ssrA.

Paper III studies the role of the ClpG/$ClpG_{GI}$ disaggregases in protein quality control and thermotolerance of *P. aeruginosa* SG17M. ClpG-type disaggregases confer superior heat tolerance through their high basal ATPase activity coupled to an efficient disaggregase activity. In addition, ClpG/$ClpG_{GI}$ bind aggregates independently without the involvement of the co-chaperone system via a unique N-terminal extension, which contrasts the established ClpB/DnaK co-chaperone system.

Paper IV describes the role of the TLPQC/LHR encoding *dna-shsp20*$_{GI}$*-clpG*$_{GI}$ operon in thermotolerance in the human commensal *E. coli* Fec10 isolate, a close homolog of *E. coli* K-12. The horizontally acquired heat tolerance locus, in particular $ClpG_{GI}$, is a major

determinant of tolerance to a lethal temperature upshift to 65 ºC. Biochemically, $ClpG_{GI}$ robustly disaggregates heat-denatured model substrates such as malate dehydrogenase (MDH) and firefly luciferase without the aid of co-chaperone factors. Moreover, $ClpG_{GI}$ shows high intrinsic basal ATPase activity and superior thermal stability compared to the ClpB disaggregase.

Paper V reports on the instant double crossover recombination frequencies upon suicide vector integration into chromosomes of the most prevalent *P. aeruginosa* clone C and PA14 strains. As a result, the genomic engineering of these prevalent clones can be facilitated by omitting the counterselection step.

Altogether, two AAA+ proteins, the FtsH protease and the ClpG disaggregases, encoded on the clone C specific genomic island PACGI-1/TLPQC and the core genome, contribute to proteostasis and confer general fitness advantages to the worldwide prevalent *P. aeruginosa* clone C.

POPULAR SCIENCE SUMMARY

Pseudomonas aeruginosa is a bacterium found almost everywhere in the environment, such as aquatic habitats and soil, but also can cause severe infections, especially in individuals with a compromised immune system. Patients suffering from Cystic Fibrosis (CF), a genetic disorder that affects, for example, the lungs, are especially susceptible to *P. aeruginosa* chronic lung infections that eventually lead to a respiratory system failure. *P. aeruginosa* is notorious for being a multi-drug resistant bacterium that is classified by the World Health Organization (WHO) as a critical priority I. The WHO goal of this classification is to raise awareness about bacteria that we are unable to treat with the currently available antibiotics and therefore urge the necessity of finding new drugs and innovative interventions. The ability of *P. aeruginosa* to form biofilms excellently makes them successful pathogens and, consequently, problematic in health care settings. The biofilm lifestyle is characterized by a multicellular aggregative form attachment to a living (e.g., lung or wound tissues) or a non-living (e.g., catheters or surgical implants) surface, and multiply to form biomass enclosed in exopolymeric substances. This extracellular matrix physically and chemically shields the bacteria from antibiotics and the host's immune cells (see Figure 2). *P. aeruginosa* is often used as a model organism to study the biofilm lifestyle of bacteria.

One distinct sub-group of closely related *P. aeruginosa* strains called clone C is globally distributed in clinical and environmental settings. To understand the spread and eventually combat successful sub-groups of pathogens, it is necessary to look for fitness traits contributing to the adaptation of strains to different niches. These fitness traits can arise from specific genes that get translated into proteins with certain physiological advantages. During their evolution, *P. aeruginosa* clone C strains acquired a particular genomic island, i.e., a foreign DNA fragment of more than 100 genes from other bacteria. This foreign genomic island called PACGI-1 encodes genes that had been predicted to be involved in stress tolerance and quality control of other cellular proteins. Consequently, a major question then was whether, and to which extent, this genomic island confers fitness advantages to one of the most prevalent sub-groups of *P. aeruginosa*.

The purpose and the physiology of any living cell are shaped by its genomic makeup that translates into the proteins with diverse functions. One important class of proteins are the proteases (the scissor proteins) specialized in chopping off aging or unwanted proteins into smaller pieces. The proteases keep the cells free from unneeded debris that can only be harmful upon accumulation. In **paper I**, we found one membrane-bound protease called FtsH2 encoded on the genomic island of *P. aeruginosa* clone C and another, highly similar but not identical, protease on the core genome called FtsH1. We found that these two proteases provided *P. aeruginosa* clone C with several fitness advantages such as proper growth and movement characteristics, intrinsic antibiotic resistance, biofilm formation and secretion of molecules needed for virulence and cell-cell communication. However, the major fitness contribution was provided by FtsH1, while the genomic island copy FtsH2 showed an additive marginal functionality to FtsH1. In **paper II,** we investigated the molecular basis behind the different degrees of the contribution of FtsH1 and FtsH2 to optimal growth of *P. aeruginosa* clone C. We found that the first 24% of the amino acid sequence of the FtsH1 protease is essential for optimal growth. On the other hand, this sequence is somewhat different in FtsH2, which explains the minor contribution of FtsH2 to fitness.

Bacteria are exposed continuously to events that eventually lead to cellular proteins to become aggregated and tangled, such as exposure to high temperatures. The process of protein aggregation can be imagined as boiling an egg where the egg proteins aggregate and solidify upon heat exposure. To prevent the toxic effect of the accumulation of aggregated proteins in the cells, bacteria developed sophisticated systems of rescuing or removing aggregates. One component of the complex rescuing system is bacterial refolding factors or disaggregases that act as officers of protein quality control by ensuring protein aggregates to be first disassembled and next properly refolded again to restore its functionality in the cell. In **paper III**, we identified a novel disaggregase encoded on the clone C specific genomic island called $ClpG_{GI}$. The high activity of the $ClpG_{GI}$ and ClpG core genome disaggregase in efficiently resolving cellular aggregates provided *P. aeruginosa* with superior heat tolerance. Unlike the classical well-studied disaggregase ClpB that requires the help of a co-protein factor called DnaK for the recruitment of aggregates, $ClpG_{GI}$ is fully active on its own.

Similarly, in **paper IV**, we found that representative strains of the human's most common commensal bacterium of the gastrointestinal tract, *Escherichia coli*, to be thermotolerant up to temperature as high as 65 ºC. The extreme heat tolerance provided by $ClpG_{GI}$ in *P. aeruginosa* and commensal *E. coli* strains raises questions about our traditional ways of using mild heat in processing raw food (e.g., smoking of meat and cheese, production of sourdough and milk powder) and sterilization below 60 ºC for heat-sensitive surgical equipment (e.g., endoscopes). Furthermore, in **paper V**, we found that clone C strains can readily recombine external genomic elements into the chromosome by double crossover recombination, which might indicate the superior ability to update the genome and acquiring genes required for survival and rapid adaptation.

In summary, the membrane-bound FtsH protease and $ClpG_{GI}$ disaggregase provide fitness advantages to the worldwide prevalent *P. aeruginosa* clone C by the degradation of proteins and dissolution of aggregated proteins and thus offer a glimpse into the molecular mechanisms of persistence and adaptation. Furthermore, this thesis suggests FtsH and/or $ClpG_{GI}$ to be potential targets to develop new (combinatorial) drugs against *P. aeruginosa* infections.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers and manuscripts

I. **Shady Mansour Kamal**, Morten Levin Rybtke, Manfred Nimtz, Stefanie Sperlein, Christian Giske, Janja Trček, Julien Deschamps, Romain Briandet, Luciana Dini, Lothar Jänsch, Tim Tolker-Nielsen, Changhan Lee and Ute Römling
Two FtsH proteases contribute to fitness and adaptation of *Pseudomonas aeruginosa* clone C strains
Front Microbiol, 2019, 10:1372

II. **Shady Mansour Kamal**, Gina D. Mawla, Tania A. Baker and Ute Römling
The N-terminus of FtsH1 but not FtsH2 is required for normal growth of *Pseudomonas aeruginosa* clone C strains
Manuscript

III. Changhan Lee, Kamila B. Franke*, **Shady Mansour Kamal***, Hyunhee Kim*, Heinrich Lünsdorf, Jasmin Jäger, Manfred Nimtz, Janja Trček, Lothar Jänsch, Bernd Bukau, Axel Mogk and Ute Römling
Stand-alone ClpG disaggregase confers superior heat tolerance to bacteria
PNAS, 2018, 115:E273-E282 (*Equal contribution)

IV. **Shady Mansour Kamal**, Annika Cimdins-Ahne, Changhan Lee, Ulrich Dobrindt, Axel Mogk and Ute Römling
A recently isolated human commensal *Escherichia coli* ST10 clone member mediates thermotolerance on a P1 phage derived IncY plasmid
Manuscript submitted

V. Changhan Lee*, **Shady Mansour Kamal*** and Ute Römling
High frequency of double crossover recombination facilitates genome engineering in *Pseudomonas aeruginosa* PA14 and clone C strains
Microbiology, 2019, 165 (7):757-760 (*Equal contribution)

PUBLICATIONS NOT INCLUDED IN THE THESIS

VI. Panagiotis Katikaridis, Lena Meins, **Shady Mansour Kamal**, Ute Römling and Axel Mogk
ClpG provides increased heat resistance by acting as superior disaggregase
Biomolecules 2019, (9), 815

VII. Elena Kashuba, Alexey A. Dmitriev, **Shady Mansour Kamal**, Ojar Melefors, Gennady Griva, Ute Römling, Ingemar Ernberg, Vladimir Kashuba and Anatoli Brouchkov
Ancient permafrost staphylococci carry antibiotic resistance genes
Microb Ecol Health Dis, 2017, 28(1)

CONTENTS

LIST OF ABBREVIATIONS

AAA	ATPase associated with diverse cellular activities
AHL	N-acyl homoserine lactone
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CF	Cystic fibrosis
ClpB/G/K	Caseinolytic peptidase B/G/K
COPD	Chronic obstructive pulmonary disease
CRISPR	Clustered regularly interspaced short palindromic repeats
ESBL	Extended-spectrum β-lactamase
GAP	GTPase-activating protein
Gm	Gentamicin
HGT	Horizontal gene transfer
HSP	Hereditary spastic paraplegia
ICU	Intensive care unit
LC-MS/MS	Liquid chromatography, Mass spectrometry
LHR	Locus of heat resistance
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
PACGI	*Pseudomonas aeruginosa* clone C genomic island
PAPI	*Pseudomonas aeruginosa* pathogenicity islands
PCA	Phenazine-1-carboxylic acid
PFGE	Pulsed-field gel electrophoresis
PVDs	Pyoverdines
PYO	Pyocyanin
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RFLP	Restriction-fragment-length polymorphism
sHsp	Small heat-shock protein
SRH	Second region of homology
T3SS	Type three secretion systems
Tc	Tetracycline
TLPQC	Transmissible locus of protein quality control
ToxA	Exotoxin A
Trx	Thioredoxin
VBMM	Vogel-Bonner minimal medium

1 INTRODUCTION

1.1 *PSEUDOMONAS AERUGINOSA* AS A NOSOCOMIAL PATHOGEN AND ENVIRONMENTAL BACTERIUM

1.1.1 Disease and habitat spectrum

Pseudomonas aeruginosa is a gram-negative, catalase- and oxidase-positive, non-fermentative, rod-shaped and monoflagellated bacterium. As an opportunistic pathogen, *P. aeruginosa* causes a wide spectrum of diseases, with up to 20% nosocomial infections with high morbidity and mortality rates (1-3). *P. aeruginosa* chronically colonizes the airways of cystic fibrosis (CF), bronchitis and chronic obstructive pulmonary disease (COPD) patients, progressively causing pulmonary damage (4). Immunocompromised individuals, patients receiving chemotherapy, intensive care unit (ICU) patients and the elderly are especially susceptible (5, 6). Other hospital-acquired infections caused by *P. aeruginosa* include bacteremia, pneumonia, as well as wound and burn infections (7). Urinary tract infections and ventilator-associated pneumonia, two of the most frequently acquired nosocomial infections, are primarily associated with contaminated indwelling devices like catheters and endotracheal tubes, respectively (2). Of note, this bacterium can also cause superficial community-acquired infections like folliculitis and the 'green nail syndrome' that occur after exposure to swimming pools or hot tubs contaminated with *P. aeruginosa* (8). Additional infections are otitis media and otitis externa (Swimmer's ear) in healthy individuals that can be, for example, acquired by diving (8). Because of its metabolic versatility and restricted nutritional requirements, *P. aeruginosa* inhabits various environmental niches such as plants, soil and natural aquatic habitats (e.g., lakes, rivers and oceans) as well as human-made niches (e.g., sewage, showers, sinks and disinfectants) (9, 10).

1.1.2 Virulence properties of *P. aeruginosa*

1.1.2.1 Biofilm

P. aeruginosa possesses diverse virulence factors, which enhances its ability to be a successful pathogen. *P. aeruginosa* usually forms biofilms in the environment. Nevertheless, biofilm formation is also a major virulence factor in disease, such as in chronic lung infections and infections associated with medical implants (11, 12). Biofilms are structured communities of bacteria enclosed in a hydrated, self-produced extracellular matrix and often attached to biotic or abiotic surfaces (13-15). Biofilm development can be divided into distinct steps from the initial attachment of motile bacteria to a biotic or abiotic surface, microcolony formation, production of extracellular matrix components including exopolysaccharides, followed by differentiation into the mature biofilm and finally dispersion of planktonic cells to colonize new territories (Figure 1) (13). The biofilm lifestyle protects the bacteria against various challenges such as environmental stresses, the host's immune response and the action of antibiotics (Figure 2) (11). In addition, in *P. aeruginosa*, quorum sensing (QS) molecules produced by the major N-acyl homoserine lactone (AHL) system (LasI/LasR) coordinate a type of biofilm formation with virulence factor expression, equally as the secondary RhlI/RhlR AHL system in combination with the synthase PqsE (16, 17). Flagella-dependent swimming motility and type IV pili-dependent twitching motility contribute to biofilm formation by affecting the initial surface sensing and attachment (18), as well as the mature three-dimensional biofilm structure (Figure 1) (19). For example, type IV

pili and flagella are necessary for forming the cap of the mushroom-like biofilm structure of *P. aeruginosa* (19).

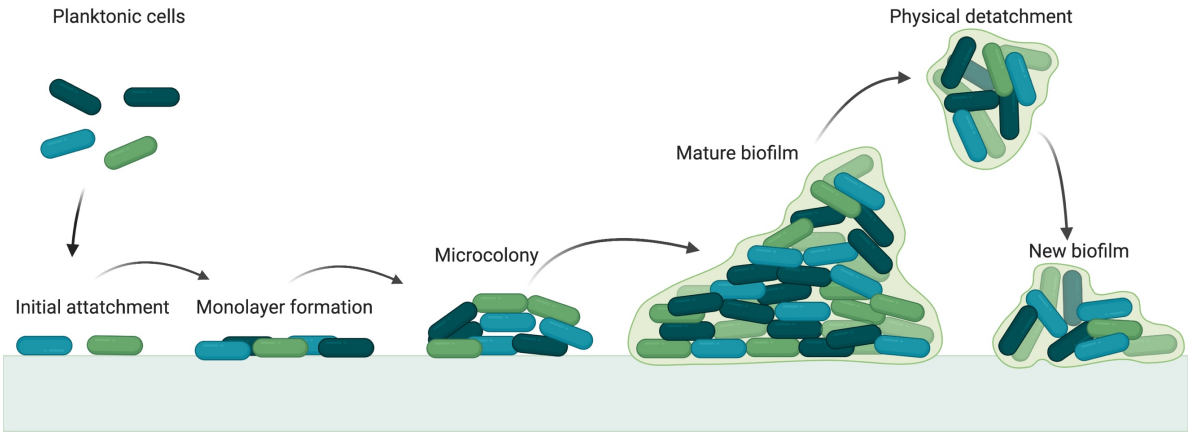


Figure 1. The developmental process of planktonic cells forming a mature biofilm from which active detachment or dispersion of motile bacteria can occur to form new biofilms.

1.1.2.2 Antibiotic resistance

P. aeruginosa is resistant to a broad spectrum of antimicrobial agents due to the different intrinsic, acquired and adaptive resistance mechanisms (20-23). The low permeability of its cell envelope confers high intrinsic resistance against some of the commonly used antibiotics such as aminoglycosides, fluoroquinolones and β-lactams (24). In addition, *P. aeruginosa* can readily acquire resistance through mutations, which lead to, e.g., reduced binding of the antibiotic to the target and/or upregulation of the efflux pumps (25). The acquired resistance can also occur via horizontal transfer of new genetic material coding, e.g., antibiotic-inactivating enzymes (22). Lastly, adaptive resistance (tolerance) is acquired through biofilm formation that confers reversible resistance through slow penetration of antibiotics, an altered microenvironment with steep oxygen and nutrient gradients and transformation of sub-populations into a dormant protective state, called persister cells, similar to spores (Figure 2) (11). Adaptive resistance can also be triggered via exposure to a sub-inhibitory concentration of antibiotics (26). Among the antibiotics effective against *P. aeruginosa* chronic lung infection in CF patients is the aminoglycoside antibiotic tobramycin (27).

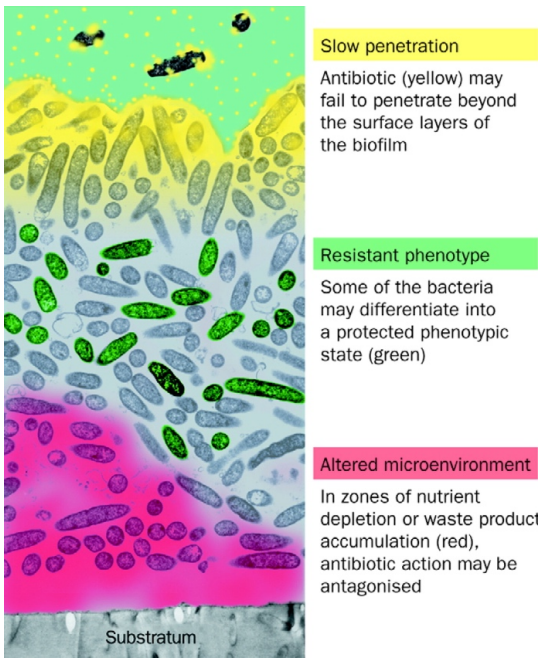


Figure 2. Mechanisms of antibiotic resistance in biofilms. The antibiotic is present in the aqueous phase at the top and the attachment of the biofilm to the substratum is at the bottom (11). Reprinted from the Lancet, 358, P. S. Stewart, J. William Costerton, Antibiotic resistance of bacteria in biofilm, 153-138, 2001, with permission from Elsevier.

1.1.2.3 Secretion systems

P. aeruginosa releases a broad spectrum of exoproteins due to the possession of diverse secretion systems (28). To carry out these activities, *P. aeruginosa* is equipped with five secretion systems, namely the secretion system(s) type one T1SS, T2SS, T3SS, T5SS and three T6SSs. These robust nano-machineries transport secreted toxins to the extracellular environment or inject effectors, in the case of T3SS and T6SS, either directly into the cytosol of a target eukaryote cell or into a competing prokaryote cell (28). The T3SS apparatus – a needle-like nano-structure – secretes at least four effector proteins, ExoS, ExoT, ExoY and ExoU, into the host cell cytosol upon close cell-cell contact (29). The effector proteins ExoS and ExoT have both GTPase-activating (GAP) and ADP-ribosyltransferase activities, which cause an antiphagocytic effect through their action on the actin cytoskeleton. ExoY accumulates cyclic AMP (cAMP) in the host cells, while ExoU is a potent patatin-like phospholipase causing rapid cell death (30). The exclusive presence of either ExoS or ExoU classifies *P. aeruginosa* strains into invasive or cytotoxic strains, respectively. Invasive strains survive and replicate inside epithelial cells and encode *exoS, exoT* and often *exoY* genes (e.g., PAO1 and clone C strain SG17M) (31). Cytotoxic strains, however, cause rapid cell death and possess the phospholipase encoding *exoU* and *exoT* and sometimes *exoY* genes (e.g., PA14) (32, 33). Similar to T3SS, T6SS delivers its effector proteins through the puncturing of other cells. Some of the effector proteins are toxic to eukaryotic cells like the phospholipase PldA and PldB, whereas others, like Tse1, Tse2 and Tse3 are toxic towards prokaryotes (34).

1.1.2.4 Secondary metabolites

The phenazine pyocyanin (PYO) is a redox-active secondary metabolite strongly associates with pathogenicity towards humans, bacteria, fungi, insects and plants. For instance, PYO exerts potent toxicity towards mammalian cells through inhibition of respiration, disruption of calcium homeostasis, misbalancing protease to antiprotease ratio and blocking epidermal cell growth (35). PYO biosynthesis is dependent on the accumulation of the QS molecules N-(3-oxododecanoyl)-L-homoserine lactone and N-butyryl-1- homoserine lactone in case cells reach a specific density (36). In *P. aeruginosa,* including clone C strain SG17M, PYO's precursor phenazine-1-carboxylic acid (PCA) is produced from two nearly identical *phz* operons each consisting of seven genes, *phz1 ($phzA_1B_1C_1D_1E_1F_1G_1$)* and *phz2 ($phzA_2B_2C_2D_2E_2F_2G_2$)* (Figure 3) (37). Subsequently, PhzM and PhzS catalyze the conversion of PCA into PYO in a two-step process (Figure 3) (38). The presence of two identical *phz* operons that are differently regulated has been suggested to maintain the balance of phenazine biosynthesis in PAO1 (39). Although *phz1* and *phz2* operons are required in PA14 to produce phenazines during the logarithmic phase of growth in liquid culture, *phz2* alone is sufficient to produce phenazine in colony biofilm (40). In addition, *phz2*, but not *phz1,* is required for host colonization in a murine model and quinolones were found to preferentially induce expression of the *phz2* operon compared to *phz1* in biofilms reiterating the necessity of conserving both operons (40).

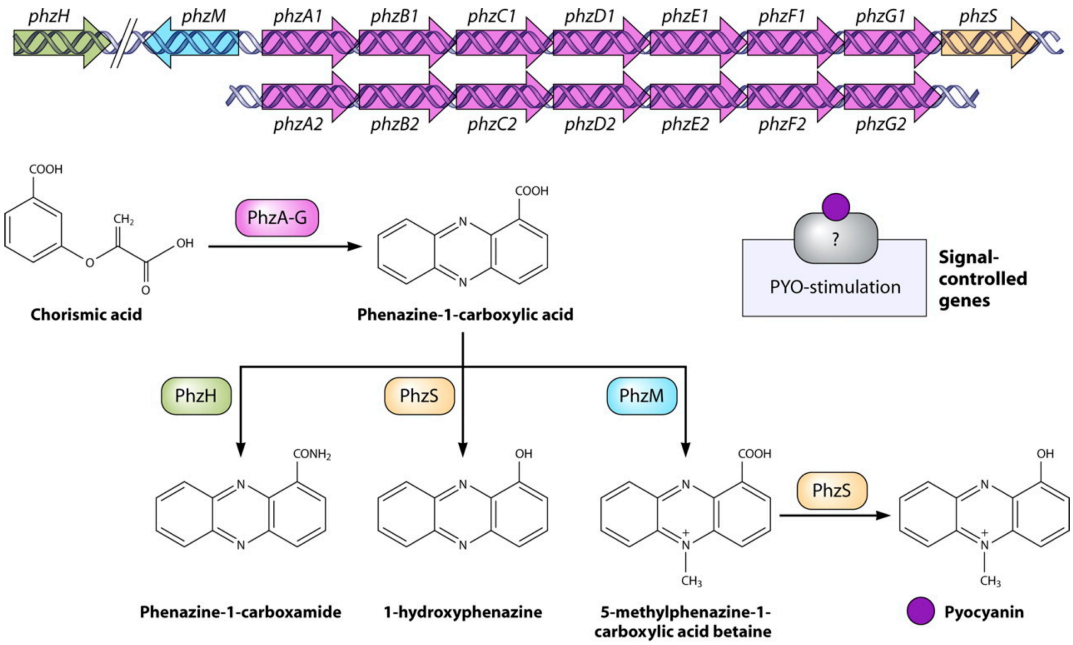


Figure 3. Biosynthesis and signaling system of pyocyanin in *P. aeruginosa* (37). Reprinted from P. N. Jimenez *et al.*, 2012, with permission from ASM.

Pyoverdines (PVDs) are a group of green fluorescent molecules, which are siderophores, powerful iron(III) scavengers and transporters, that have a dual-role in iron uptake and pathogenesis (41, 42). Furthermore, PVDs act as QS molecules that activate – through a cascade of processes – the production of exotoxin A (ToxA) and other proteases involved in virulence (43, 44). The PVD structure shows chemical diversity between different *Pseudomonas* species and even within a species (45); for example, *P. aeruginosa* has three different distinct PVD groups with a conserved fluorescent dihydroxy quinolone chromophore group (41). PVDs can be identified by their UV spectrum profile due to the chromophore in their structure; they absorb UV light at 380 nm and emit fluorescence at 447 nm (46).

1.2 POPULATION STRUCTURE OF *P. AERUGINOSA*

1.2.1 Population structure of *P. aeruginosa* unraveled by genome sequencing

Prior to the whole-genome sequencing era, alternative molecular typing approaches were applied to unravel the population structure of *P. aeruginosa*. For example, a macrorestriction fingerprint of the genome of *P. aeruginosa* strains is obtained by restriction digestion of the genome with a rare-cutting enzyme, such as *Spe*I. Separation of obtained fragments by Pulsed-Field Gel Electrophoresis (PFGE) renders a unique pattern of high molecular weight DNA fragments – a distinct basic fingerprint for each clonal group (47, 48). Other approaches used are the hybridization of distinctly labeled DNA fragments onto a microarray (49) and Multilocus Sequence Typing (MLST) by comparing the nucleotide sequences of several different housekeeping loci (50, 51).

In the post-genomic era, when whole-genome sequencing became more affordable, high-quality genome sequences of an increasing number of *P. aeruginosa* isolates of different origins have been made available. Consequently, refinement of the population structure of *P. aeruginosa* by defining, for example, the core genome (genes present in all *P. aeruginosa* isolates), the accessory genome (genes present in a fraction of *P. aeruginosa* isolates) and the gene reservoir of the species (pan-genome) could be initiated (52). B. Valot *et al*., (2015) refined the core genome borders of *P. aeruginosa* from reanalyzing 17 reference strains (53). J. Mosquera-Rendón *et al*., (2016) expanded the pan-genome analysis of the *P. aeruginosa* population to include 181 strains to understand the genetic variation and its correlation with vaccine design against *P. aeruginosa* (54). Whereas L. Freschi *et al*., (2019) has updated the pan-genome of *P. aeruginosa* to consist of 54,272 genes whereby only 665 genes were found to be core genes by comparing 1,311 *P. aeruginosa* isolates (55). On this basis, the population was classified into two major groups containing most of the isolates and three smaller groups with the evolution of resistance and virulence genes linked to this classification (55). Consistently, a recent analysis of 739 *P. aeruginosa* genomes found the two largest groups of *P. aeruginosa* isolates in the population predominantly encoded either *exoS* (group A) or *exoU* (group B), reflecting a restricted gene flow between the two groups, but not within the groups (Figure 4) (56).

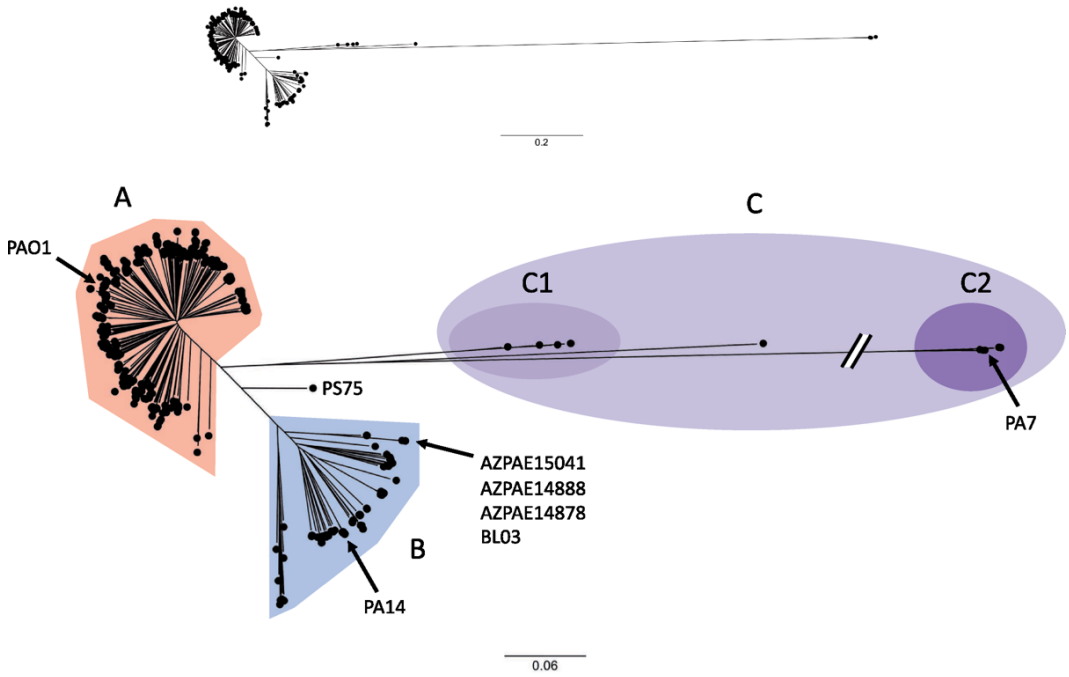


Figure 4. Population structure of whole-genome sequences of 739 *P. aeruginosa* isolates from diverse sources (56). Reprinted from E. A. Ozer *et al*., 2019, under the terms of the Creative Commons CC BY license.

1.2.2 Genome plasticity of *P. aeruginosa*

Evolutionary, bacteria undergo constant genetic modifications to cope with the adaptive pressure from changing environments. The process of diversification takes place through different mechanisms, from mutation of single nucleotides, to intragenomic reshuffling, intergenomic transfer of chromosomal DNA and acquisition of foreign DNA segments from another organism through a process termed horizontal gene transfer (HGT) (57).

Horizontal transfer of DNA segments results in the integration of 'so-called' genomic islets (<10 kb) or islands (>10 kb) at distinct loci called Region of Genomic Plasticity (RGP) (58). Genomic islands encode gene products that provide advantageous properties such as elevated pathogenicity, antibiotic determinants, genes related to metabolism and/or fitness-related genes (59). For instance, two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) of 108 kb and 11 kb in size, respectively, have been found in the highly virulent PA14 strain that were absent from the less virulent PAO1 strain (60). The recently performed whole-genome sequencing initiatives allow a more detailed analysis of changes down to the nucleotide levels in different *P. aeruginosa* strains. The accessory genome, on the other hand, evolves faster than the average genome (61).

1.2.3 The most common *P. aeruginosa* clones – with C strains as the most common clonal group

Throughout the years, *P. aeruginosa* strains PAO1 (62) and subsequently, PA14 (63) have been, and still are, extensively used in molecular and genetic studies and therefore imprinted our view of the *P. aeruginosa* species. A clone or a clonal group consists of closely related isolates of a bacterial species that are significantly more related to each other than to other isolates, which is reflected by the fact that they group together when using different typing methods (64, 65). While the PAO1 strain belongs to a more uncommon clonal group, the PA14 strain belongs to the second most abundant clonal group. The most common clonal group in the *P. aeruginosa* population is clone C strains (66, 67).

The dominance of clone C strains has been first recognized by their ability to infect CF patients worldwide successfully (48, 68). Subsequently, clone C strains have been found to infect patients with chronic obstructive bronchitis (69), cause urinary tract infections (70) and a broad spectrum of other diseases. In addition, clone C strains dominate natural and artificial aquatic habitats (68, 71-73). Their ability to colonize different reservoirs does not only point towards their ability to tolerate different environments but also to possess and adapt fitness traits required to thrive in different environmental niches. These fitness traits that enable the prevalence of clone C worldwide can be encoded on the core genome, on the accessory genome or both (74).

1.2.4 The accessory genome of *P. aeruginosa* clone C strains

Chromosomal plasticity of clone C strains of different origin was first discovered when the genomic fingerprint created by macrorestriction digestion was analyzed by PFGE, followed by the construction of the physical genome maps of strains of environmental and clinical origin (67, 75). These analyses showed that clone C strains, through acquisition and deletion of genetic information (genomic islands and islets), are more variable than strains of clones that showed a more restricted distribution. As a hallmark of clone C, a 103.5 kb plasmid called pKLC102 is present in almost all clone C strains and integrated into the chromosome of cystic fibrosis isolates (76, 77). Other mobile genetic elements have been distinctively acquired by clone C strains like the genomic island PAGI-2 and PAGI-3 (78).

In order to investigate the successful prevalence and transmission of clone C strains, the whole-genome sequence was determined for one representative clone C member called SG17M, isolated from a river in Germany (67, 79, 80). Comparative genome analysis between SG17M and the well-studied non-clone C strains like PAO1 revealed the acquisition of a clone C specific genomic island (PACGI-1) of 86 kb with more than 100 genes

integrated into a clustered regularly interspaced short palindromic repeats (CRISPR) locus (Figure 5) (74). The clone C-specific genomic island PACGI-1 harbors the transmissible locus of protein quality control (TLPQC), also known as Locus of Heat Resistance (LHR) in other bacteria (81, 82) (see below). TLPQC encodes stress response and protein quality control related gene products such as a third homolog of a superoxide dismutase (SOD), a fourth homolog of thioredoxin (Trx), a DNA damage repair-related protein (RadC) and a second copy of the essential FtsH protease (74). Most importantly, TLPQC harbors the tri-gene cluster *dna-shsp20$_{GI}$-clpG$_{GI}$* that confers tolerance to lethal heat-shock to clone C members (74, 83). ClpG (known as ClpK in *Klebsiella pneumoniae*) is a major determinant of thermotolerance in *K. pneumoniae* (81), food-borne *E. coli* (82) and other bacterial species.

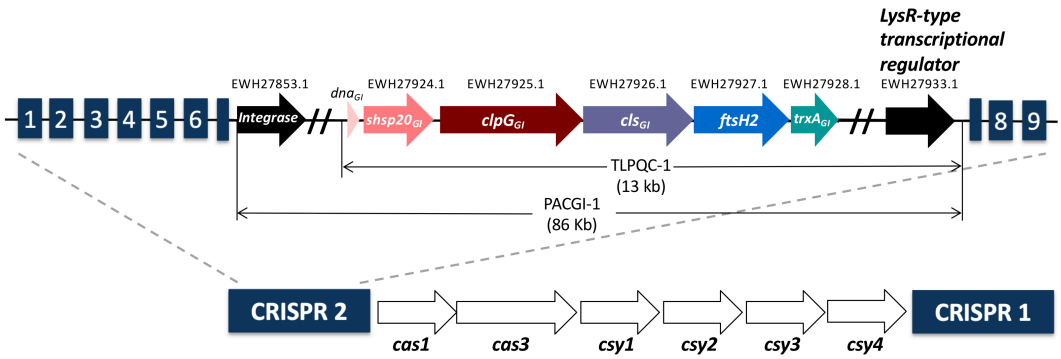


Figure 5. Integration of clone C specific genomic island (PACGI-1) into the 7th and the 8th palindromic repeat of CRISPR 2 locus of SG17M strain. Figure is adapted from (74) and (84).

1.3 POPULATION STRUCTURE OF *ESCHERICHIA COLI*

1.3.1 Overview over the population structure of *E. coli*

E. coli is a gram-negative commensal bacterium that predominantly inhabits the gut of vertebrates, but is also a pathogen capable of causing intestinal and extraintestinal infections. The genomic structure of the *E. coli* population in the pre-sequencing era was classified according to serotypes using the combined distinction of O (somatic), K (capsular) and H (flagellar) antigens (85, 86). As the serotype and the pathogenicity were non-randomly associated, it described the population as stable clones with few genomic recombinations (87). Subsequently, multilocus enzyme electrophoresis (MLEE) classified the population into four main groups (A, B1, B2 and D) and two accessory groups (C and E) based on 38 housekeeping enzymes (88, 89). Other techniques extended the MLEE analyses further, such as the random amplified polymorphic DNA (RAPD) and restriction-fragment-length polymorphism (RFLP) of the ribosomal RNA genes and PFGE to confirm the clonal concept of the population (90-92). Three specific gene markers were identified for the four main phylogroups (A, B1, B2 and D) and a simple combinatorial PCR approach is used until today to grossly classify strains within the *E. coli* population (93). With the increased availability of MLST data of *E. coli* isolates, the phylogroup classification was revisited and updated to eight phylogroups, seven belonging to *E. coli sensu stricto* (A, B1, B2, C, D, E, F) and one to *Escherichia* clade I (94). A recent study has performed a systematic review on 217 studies that performed multilocus sequence typing or whole-genome sequencing of *E. coli* isolates (95). *E. coli* isolates recovered from the gut and extraintestinal infection were classified into 20 major sequence types with the multi-drug resistant ST131 as the most frequent sequence type (95).

1.3.2 Commensal *E. coli* strains including the model strain *E. coli* K-12

E. coli is part of the mammalian gut microbiome and the most common aerobe in the lower intestine of mammals (96). *E. coli* grows in the thin layer of the mucus lining in the gut as a multispecies biofilm (97, 98). Inhabitation of *E. coli* to the human gut begins at birth when *E. coli* colonizes the infant gut epithelium acquired mostly from the mother and the surroundings (99). The *E. coli* community colonizing the gut, which is composed of long- and short-term inhabitants, is shaped by the host's diet and exposure to antibiotics (100, 101). The human host aids *E. coli* establishment in the gut by secreting immunoglobulin A that promotes the formation of *E. coli* biofilm on the intestinal mucosa (102). In return, *E. coli* provides the host with vitamin K and vitamin B12 (103, 104) as well as excluding colonization of the gut by pathogens (97). Therefore the relationship between *E. coli* and the human host is mutualistic. However, it is worth noting that *E. coli* can cause intra and extraintestinal infections like diarrhea, colitis, bacteria and urinary tract infections in immunocompromised and even in healthy individuals (105, 106).

E. coli K-12 is a commensal strain that belongs to the ST10 MLST type, which has been the genetic reference strain since 1921 (107). The K-12 strain and its derivatives have been widely used by scientists as a model organism to deliver groundbreaking discoveries in bacterial physiology, basic biology and genetics (108). Of note, *E. coli* K-12 is closely related to the first commensal *E. coli* strain NCTC86 discovered by Theodor Escherich in 1886 (109, 110).

1.3.3 The locus of Heat Resistance in bacteria

The Locus of Heat Resistance (LHR), synonym to TLPQC, a genomic island of up to 19 kb found in several bacteria, encodes gene products that are involved in protein folding and turnover, thus providing extreme thermotolerance, resistance to pressure and oxidative agents (82, 83, 111). In several *Klebsiella pneumoniae* clinical isolates, in particular, the multi-drug resistance lineage C132-98 isolated from critical ICU infections, the LHR is encoded on a conjugative plasmid. In this context, LHR contains a determinative component ClpK (ClpG) disaggregase that is responsible for a remarkable thermotolerance, which enables *K. pneumoniae* to thrive in the hospital environment through, for example, surviving mild heat sterilization below 60 ºC as used for thermo-labile endoscopes (81). Similarly, some Enterobacteriaceae family members, including the opportunistic pathogen *Cronobacter sakazakii* associated with a severe infection in neonates, were found to harbor the LHR contributing to a prolonged thermotolerance at 58 ºC which can be linked to contamination of powdered infant milk formula (112). The beef isolate *E. coli* AW1.7 harbors the LHR locus and therefore surviving the ''well-done'' temperature of 70 ºC (113). Also, other heat-resistant *E. coli* isolates from clinical and environmental origins showed the acquisition of LHR (82). Another food-borne pathogen of economic impact and raising concern over food safety is *Salmonella enterica* Serovar Senftenberg ATCC 43845 that harbors two thermotolerance loci (114). Recently, the thermotolerant raw milk cheese isolate *E. coli* FAM21805 was found to harbor two distinct LHR variants, one of them encoded on a highly transmissible plasmid (115). The two LHR operons were also identified in extended-spectrum β-lactamase (ESBL)-producing *E. coli* C604-10 that cooperatively provide thermo-protection (116). In this thesis, we show that *E. coli* strains with high thermotolerance, one of them closely related to *E. coli* K-12, persist in the human gastrointestinal tract (Kamal *et al*., manuscript IV, submitted).

1.4 PROTEIN HOMEOSTASIS IN BACTERIA

1.4.1 General molecular mechanisms of protein homeostasis

Bacteria have developed a variety of sophisticated systems to maintain the structure and the function of all cellular proteins under physiological and stress conditions. Different chaperones and proteases coordinate their activities to maintain protein homeostasis by ensuring proper folding of the newly synthesized polypeptides and removing the misfolded entities. These misfolded polypeptides can be disaggregated and subsequently refolded or eventually degraded by proteases (117).

Proper folding of the newly synthesized proteins is ensured by the chaperone systems DnaK/DnaJ/GrpE, the chaperonines GroEL/GroES and the chaperone trigger factor (TF) (118, 119). Upon exposure to environmental stressors such as temperature/pH/oxygen shift, osmotic shock and exposure to reactive compounds, different chaperones cooperate to maintain the protein integrity such as small heat-shock proteins (sHsp), the Hsp100 AAA+ (ATPase associated with diverse cellular activities) disaggregase ClpB and Hsp70 (DnaK) (120-124). Consequently, these chaperone systems form a functional triad of protein homeostasis maintenance, from holding (e.g., IbpA/sHsp), folding (e.g., DnaK/DnaJ/GrpE) to disaggregating chaperones (e.g., ClpB) in order to efficiently recover aggregated proteins (83, 125). Furthermore, different AAA+ proteases irreversibly degrade misfolded or misassembled proteins as another way to maintain protein quality when the disaggregation systems are insufficient (Figure 6) (126, 127).

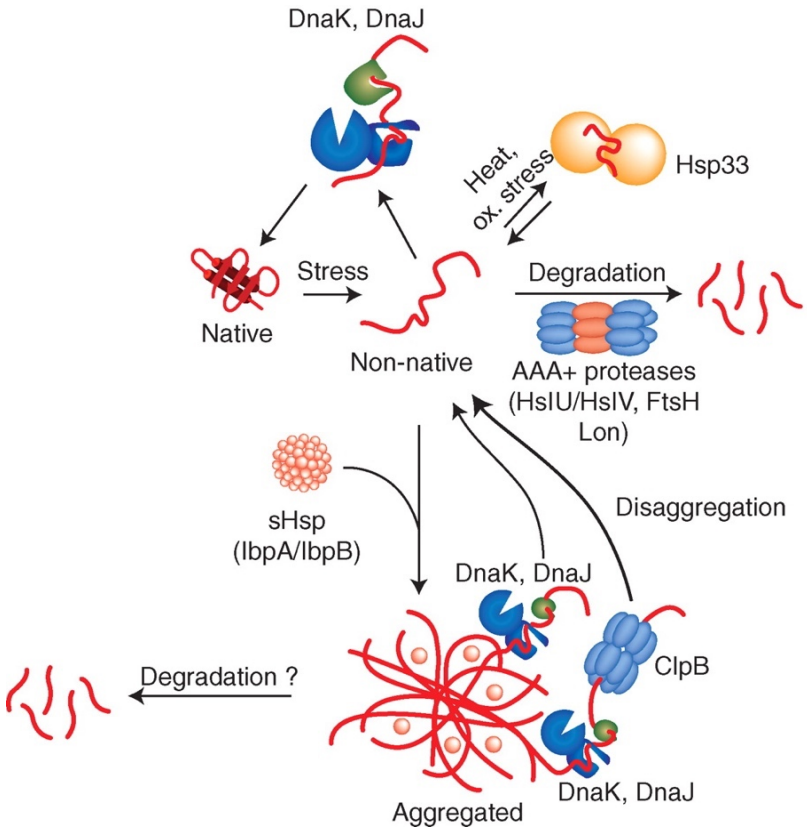


Figure 6. Bacterial protein quality control systems during environmental stressors like heat and oxidative stress (117). Reprinted from A. Mogk *et al.,* Integrating protein homeostasis strategies, 2011, Vol. 3, 3:a004366, with permission from Cold Spring Harbor Laboratory Press.

1.4.2 The AAA+ domain

AAA+ superfamily proteins are part of the diverse P-loop NTPases class that is widespread in all domains of life (128). The hallmark of these proteins is the AAA+ module of 200-250 amino acids that utilizes energy derived from nucleotide hydrolysis for macromolecule remodeling in target proteins (129, 130). The AAA+ module contains the conserved nucleotide-phosphate binding domain referred to as P-loop or Walker A motif ($GX_4GK[S/T]$), where the lysine binds β- and γ-phosphates of bound ATP, while the threonine/serine coordinates the Mg^{2+} ion (131). Downstream of the Walker A motif locates the Walker B motif (XXXXDE), where X is any hydrophobic residue (132, 133). The conserved aspartate residue in the Walker B motif coordinates Mg^{2+} ion binding, whereas the glutamate residue primes a water molecule for the nucleophilic attack of the γ-phosphates of the bound ATP by proton abstraction (134). The hydrolysis of the ATP leads to a relative movement of the N- and C- terminus of the AAA+ modules generating a mechanical force applied to the remodeling of the bound substrates (130).

1.4.3 Disaggregating chaperones

The Hsp100 family member chaperone ClpB is present in most bacteria. ClpB is an AAA+ disaggregase/unfoldase with two ATPase domains involved in energy-driven ATP hydrolysis necessary for aggregate unfolding. The ClpB chaperone cannot work independently but requires the DnaKJE co-chaperone system that binds to the aggregates and escorts it to the ClpB chaperone to stimulate the ATPase activity of ClpB (Figure 7A) (135, 136). The bi-chaperone system ClpB/DnaKJE, assisted by small Hsps, is considered to be the major disaggregating chaperone system upon lethal heat stress in *E. coli* and other bacteria (121-123, 137).

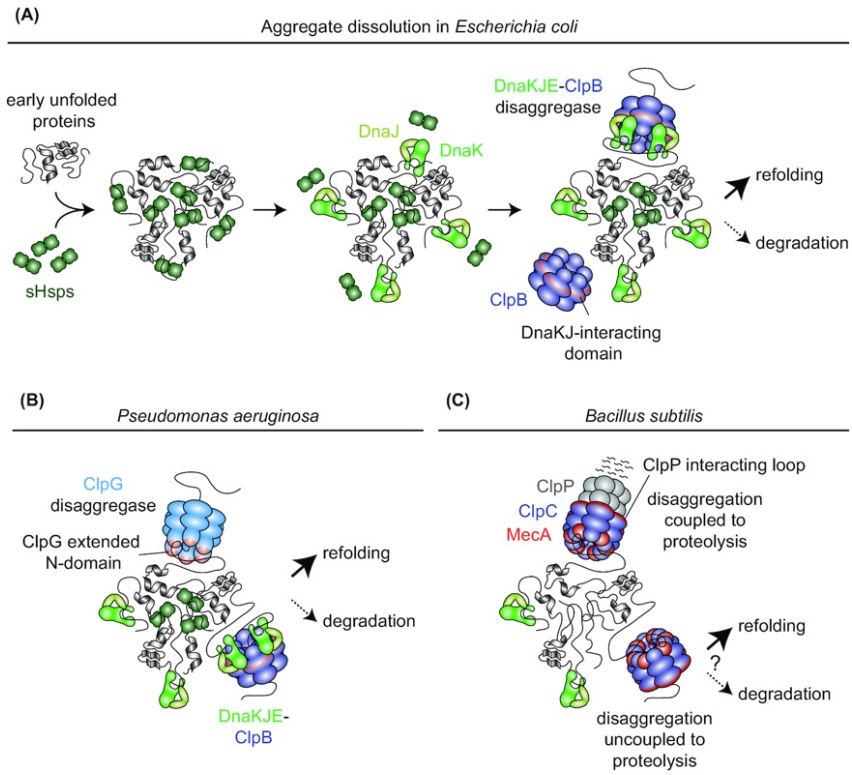


Figure 7. Different strategies for aggregate solubilization or degradation in different bacterial species (137). Reprinted from F. D. Schramm *et al.*, protein aggregation in bacteria, FEMS Microbiol Rev, 2020, Vol 44, Issue 1, with permission from Oxford University Press.

In addition to the bi-chaperone system ClpB/DnaKJE, *P. aeruginosa* has a novel, horizontally transferred Hsp100 chaperone, the ClpG protein, which is highly homologous to ClpB (74). ClpG is a disaggregating chaperone of the AAA+ protein family that has two potent ATPase domains flanking a distinct middle domain (M-domain). *P. aeruginosa* clone C strains have two copies of ClpG, one encoded on the core genome (ClpG) and one encoded by the TLPQC locus on the genomic island PACGI-1 ($ClpG_{GI}$) with 76% sequence homology. ClpB protein, related to ClpG/$ClpG_{GI}$ in the primary sequence and structure, requires the Hsp70 protein DnaK for aggregated protein binding and ATPase activation. Whereas, ClpG/$ClpG_{GI}$ have high basal ATPase activity and do not require DnaK for aggregate delivery. Instead, the ClpG class disaggregases bind aggregates via a unique N-terminal extension that is absent in ClpB, conferring superior heat tolerance (Figure 7B) (138). Furthermore, ClpG uniquely recognizes 'tighter' aggregates formed at higher temperatures and protein concentrations, which are only poorly processed by the ClpB/DnaK system (139).

Some gram-positive bacteria (e.g., *Bacillus subtilis*) lack ClpB and ClpG homologs and instead depend on Hsp100 unfoldases like ClpC containing a ClpP interacting loop (140, 141). Similar to ClpB, the activity of ClpC essentially requires adaptor proteins such as MecA that binds to the aggregates and escorting it to ClpC while stimulating its activity (142). Although the ClpC/MecA system displays unfoldase activity *in vitro*, the ClpP interacting loop in ClpC allows docking of ClpP that leads to disaggregation directly coupled to ATP-dependent proteolysis (Figure 7C) (137, 142).

1.4.4 AAA+ proteases

As exemplified by ClpC, other components involved in protein homeostasis are the AAA+ proteases that contain one or more ATPase domains involved in energy-driven ATP hydrolysis, required for substrate unfolding (the AAA+ disaggregase/unfoldase domain such as ClpA or ClpX) and subsequent translocation to a protease domain. For example, *E. coli* encodes five AAA+ proteases Lon, ClpXP, ClpAP, HslUV and FtsH (126), whereby the Lon protease is responsible for approximately 50% of *in-vivo* proteolysis (127, 143). The AAA+ disaggregase/unfoldase domain can be covalently coupled to the protease domain, as in FtsH and Lon. Alternatively, AAA+ disaggregase/unfoldase and protease can be two distinct proteins, such as HslUV, ClpXP, ClpAP and ClpCP (Figure 8) (127, 144).

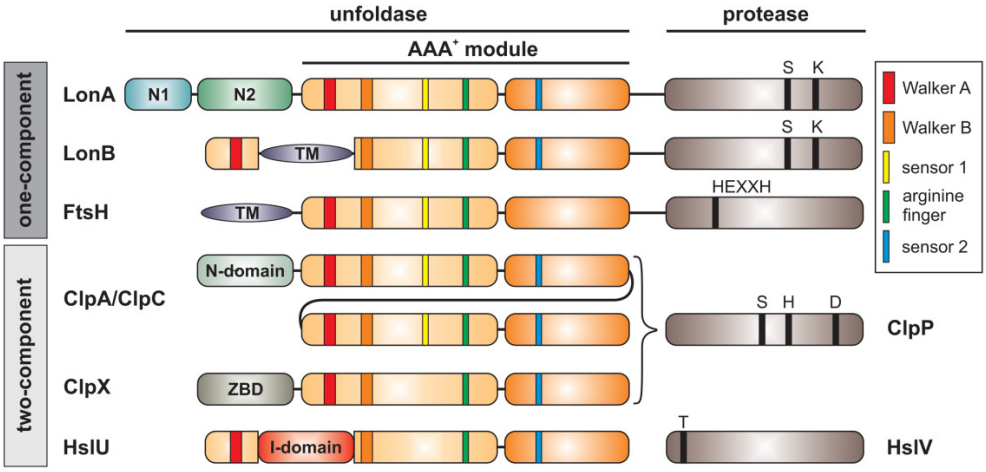


Figure 8. The domain structure of AAA+ proteases in bacteria (144). Reprinted from L. M. Bittner *et al.*, ATP-dependent proteases in bacteria, Biopolymers, 2016, Vol 105, Issue 8, with permission from John Wiley and Sons.

Some of these proteases play a role in antibiotic resistance, motility, biofilm formation and metabolism. For example, Lon and ClpP are involved in *P. aeruginosa* tolerance against the fluoroquinolone ciprofloxacin (145, 146) as well as motility (147, 148). In addition, the Lon protease negatively regulates the QS system RhlI/RhlR that is linked to pyocyanin biosynthesis by degrading LasI, leading to repression of LasR/LasI. Therefore, the deletion of the Lon protease leads to the accumulation of pyocyanin (149).

1.4.5 Mechanism of action of AAA+ proteases

Degradation by AAA+ proteases takes place through four consecutive steps. The first step is the recognition of the substrate by the ATPase domain directly or indirectly. Direct recognition is based on a short recognition motif (degron) in the substrate with a specific sequence and/or length and/or chemical property (150). For example, ClpXP and FtsH identify the ssrA tag, a C-terminal polypeptide of 11 amino acid residues (151, 152), while Lon protease recognizes a degron, rich in hydrophobic residues in unfolded proteins, but also specific motifs in stable native proteins (153). Substrate recognition by AAA+ proteases can also occur through adaptor proteins (154). For example, the adaptor protein ClpS binds to specific amino acids at the N-terminus of a substrate and delivers it towards ClpAP for degradation (155, 156). The second and third steps are unfolding and translocation of the linear substrate into the proteolytic chamber that is constituted of hexamers as a barrel-like structure of disaggregase/protease monomers; these two steps require energy, driven by ATP hydrolysis of the AAA+ disaggregase/unfoldase domain. The last step involves ATP-independent degradation at the active site of the protease (Figure 9) (127, 144, 157).

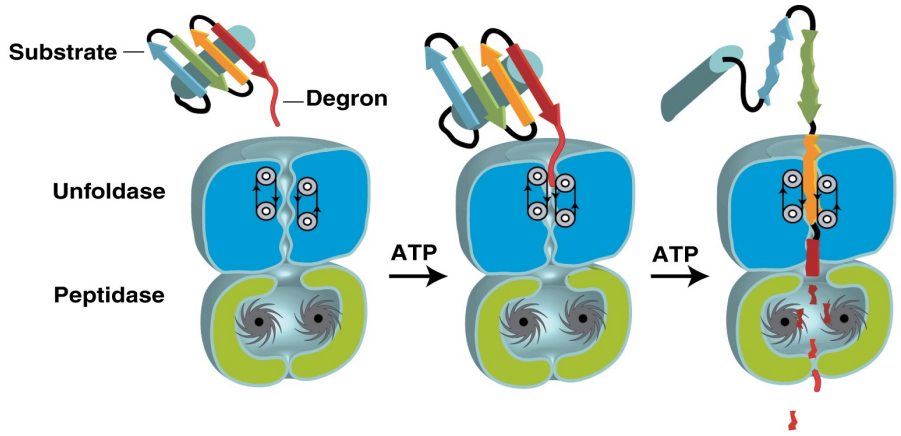


Figure 9. Degradation process by AAA+ proteases. AAA+ unfoldase and peptidase domains are indicated (127). Reprinted from R. T. Sauer, T. A. Baker, AAA+ Proteases: ATPFueled Machines of Protein Destruction, Annu Rev Biochem, 2011, Vol 80, with permission from Annual Review.

1.5 FTSH PROTEASE

1.5.1 Features of the FtsH protease

The FtsH protease belongs to the AAA+ protease family and is universally conserved between eubacteria, mitochondria and chloroplasts, indicating the functional importance of this protein (158, 159). Among the five AAA+ proteases in *E. coli*, FtsH is the only membrane-bound protein and is required for viability (160). Inactivation of *ftsH* in *Bacillus subtilis* and yeast AAA protease causes pleiotropic phenotypes (161, 162), whereas mutations in the human mitochondrial AAA proteases leads to Hereditary Spastic Paraplegia (HSP) (163).

FtsH is anchored to the inner cell membrane via the N-terminal domain as homo-hexameric complexes (164-169). The cytosolic part of the protease contains two conserved active domains, the AAA+ ATPase and the Zn^{2+}-dependent metalloprotease domain (Figure 10) (170).

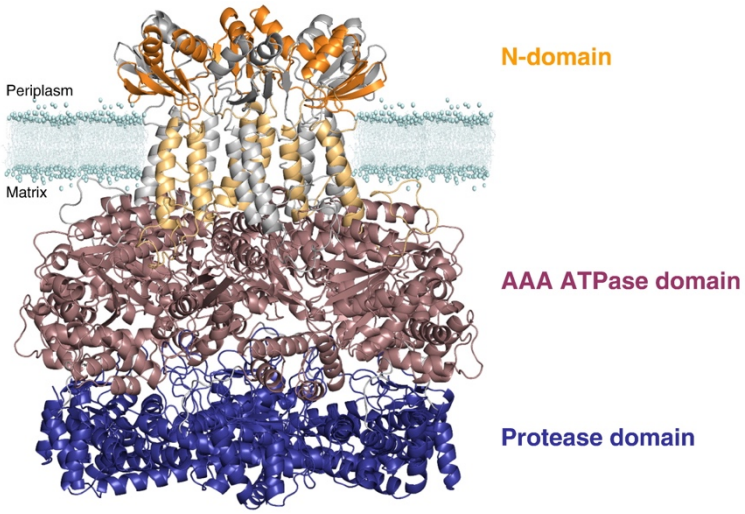


Figure 10. Structural model of the *E. coli* AAA+ protease FtsH (171). Reprinted from F. Scharfenberg *et al.*, 2015, under the terms of the Creative Commons CC BY license.

The ATPase domain hydrolyzes ATP to unfold and translocate the substrates through the central pore into the proteolytic chamber (127). Accordingly, the AAA+ ATPase domain contains the Walker A/Walker B motifs that are required for nucleotide binding, metal ion coordination and ATP hydrolysis (172) and the pore motif that is necessary for coupling unfolding with translocation (167). Furthermore, the AAA+ ATPase contains the second region of homology (SRH) that is critical for ATPase activity and to distinguish AAA family members from other Walker A/B motif-containing ATPases (Figure 11) (159, 173, 174). FtsH is an M41 metallopeptidase that requires a divalent metal cation (zinc) for peptidase activity (170). The zinc ion is held in place by two conserved histidine residues in the HEXXH motif (X: any amino acid) and a downstream aspartate residue as a third coordinating residue. The ionization of the zinc-bound water leads to the nucleophilic attack on the peptide bonds of the substrates via zinc hydroxide (169, 175).

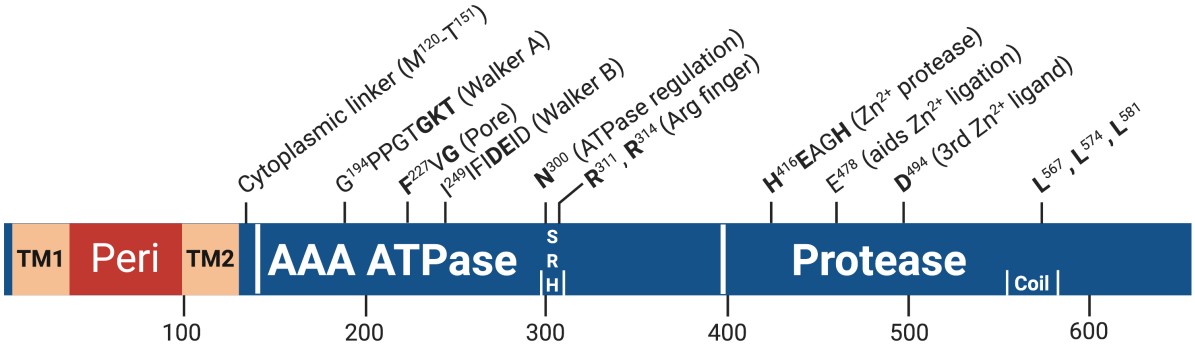


Figure 11. Horizontal representation of *P. aeruginosa* FtsH1 from N-terminal (left) to C-terminal (right). The transmembranes, periplasmic and cytoplasmic domains are shown in different colors. The functional motifs are indicated. The figure is created based on (159).

1.5.2 The N-terminal domain of FtsH

The N-terminal domain is essential for the topology and functionality of FtsH proteases (176, 177). It consists of two transmembrane helices flanking a periplasmic domain followed by a glycine-rich cytoplasmic linker connecting the N-terminal domain to the AAA+ ATPase domain (159). Structurally, the two transmembrane helices anchor FtsH to the inner cell membrane and drive homo-oligomerization together with the periplasmic domain (165, 166, 178). Functionally, the transmembrane helices of the N-terminal domain contribute to the proper ATPase and peptidase activity of FtsH *in vivo* and *in vitro* (176-179). Two membrane-anchored periplasmically-exposed proteins called HflK and HflC interact with FtsH via its periplasmic domain, forming a large holoenzyme complex in the inner membrane that modulates substrate specificity (166, 180, 181). Unlike the AAA+ ATPase and peptidase domain of the AAA+ metalloprotease core domains, the N-terminal region of the FtsH protease seems to diverge substantially (171, 182).

1.5.3 Functions of the FtsH protease

1.5.3.1 Membrane- and periplasm-related functions/properties

The unique location of FtsH in the inner cell membrane allows interaction with periplasmic, membrane-standing and cytoplasmic substrates. FtsH controls the membrane protein quality by degrading uncomplexed membrane proteins such as the T2SS translocon protein SecY in case of failed pairing with the SecE to form a translocase (183, 184). Similarly, FtsH degrades the subunit alpha of the F_1F_0 ATP synthase complex (184). Besides its function as a protease, FtsH in concert with HflK, HflC and YidC, acts as a chaperone to maintain the integration of inner membrane proteins (Figure 12) (185-187). In addition to its regulatory and proteolytical properties in the cell membrane, FtsH processes and translocates the cytotoxic C-terminal domain of the tRNAase toxin colicin D from the periplasm into the cytoplasm in collaboration with LepB (Signal peptidase I), an essential element of cellular secretion machinery (188-190) (Figure 12). Furthermore, in *Salmonella typhimurium,* FtsH negatively regulates the virulence factor membrane protein MgtC required for survival inside macrophages (191).

1.5.3.2 Cytoplasmic-related functions/substrates

FtsH protease can act on soluble substrates in the cytoplasm. FtsH degrades the LpxC and WaaA (KdtA) enzymes that are involved in the biosynthesis of the hydrophobic anchor of the LPS called lipid A (160, 192, 193). Consequently, FtsH controls the balance between lipopolysaccharide (LPS) and phospholipids – key components of the outer membrane and crucial for cell survival. In addition, together with the ClpAP and ClpXP proteases, FtsH clears the cytoplasm from abnormal polypeptides tagged with the degradation signal ssrA added to truncated mRNAs (151, 152). FtsH is also involved in regulating gene expression linked to heat-stress through rapid degradation of the heat-shock transcription factor σ^{32} (RpoH) at non-stress temperatures (194-196). FtsH also plays an indispensable role in targeting crucial viral proteins. For example, during bacteriophage infection, FtsH contributes to the decision between lysis and lysogeny through keeping the balance of the transcriptional activator of bacteriophage λ protein cII (Figure 12) (197, 198).

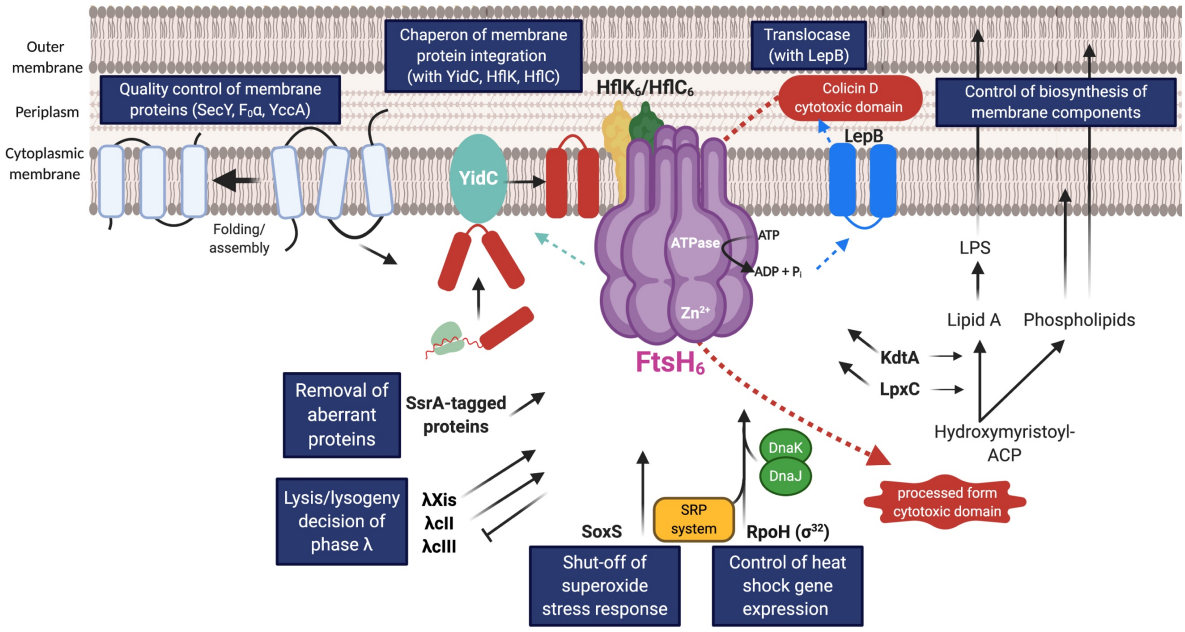


Figure 12. Overview of the diverse functions of the FtsH protease in *E. coli*. The figure is adapted and modified from (199).

1.5.4 Regulatory proteins of the FtsH protease

Proteases often use accessory/adaptor proteins to aid in the degradation of substrates (154). The substrate specificity and the catalytic activity of FtsH towards some substrates are fine-tuned by regulatory proteins and substrate-specific modulators, as summarized in Table 1.

Table 1. List of regulatory and substrate-specific proteins of FtsH proteases.

Regulatory protein/substrate-specific modulator	Function	Organism	Reference(s)
HflK/HflC	Negative regulation of proteolysis of the membrane substrate SecY *in vivo* and *in vitro* and the cytoplasmic substrate cII *in vitro*	*E. coli*	(184, 197)
HflD	Sequestering and presenting cII for degradation	*E. coli*	(200)
DnaK chaperone system and Signal Recognition particle	Escorting the cytoplasmic regulatory transcription factor RpoH (σ^{32}) for degradation	*E. coli*	(201, 202)
LapB (YciM) and WaaH	Stimulate the rapid turnover of LpxC	*E. coli*	(192, 203, 204)

LepB (Signal peptidase I)	Required for processing and translocation of colicin D across the membrane	*E. coli*	(190)
MgtR	Promotes the degradation of the virulence factor MgtC	*Salmonella enterica*	(191)
YqgP	Serves as an adaptor for FtsH to cleave the major magnesium transporter MgtE	*Bacillus subtilis*	(205)
YidC/HflK/HflC	Act as a chaperone with FtsH for membrane protein integration	*E. coli*	(185-187)

While the FtsH protease has been extensively characterized in *E. coli*, it remains poorly investigated in *P. aeruginosa* (206-208). Of note, *P. aeruginosa* clone C strains have two homologous copies of *ftsH*, one on the core chromosome and another one on the genomic island PACGI-1, namely *ftsH1* and *ftsH2*, respectively. Also, other organisms have more than one FtsH copy, like the cyanobacterium *Synechocystis*, which has four copies (209) and *Arabidopsis* chloroplasts having nine copies involved in photosynthesis (210).

1.5.5 Substrate recognition mechanisms by FtsH

FtsH is involved in processing a variety of diverse substrates and is thus involved in various cellular activities, yet the recognition logic of the substrates is not fully understood (150). Even for the sole FtsH protease of *E. coli*, there is no universal degron sequence that is recognized by the FtsH protease. For example, the general recognition C-terminal ssrA-tag (AANDENYALAA) directs the mistranslated proteins to AAA+ proteases including FtsH (151, 211). Also, the degron of LpxC, LAXXXXXAVLA (X: any amino acid), contains several non-polar residues (and thus resembles the ssrA-tag sequence) and is located at the C-terminus (212, 213). The degron, however, can also be located at the N-terminus as in the case of YfgM, where the degron consists of the first 14 N-terminal amino acids (MEIYENENDQVEAV-) (214). The N- and C-terminal ends of RpoH, however, are not necessary for degradation as the degron is located internally in the protein (Figure 13) (215).

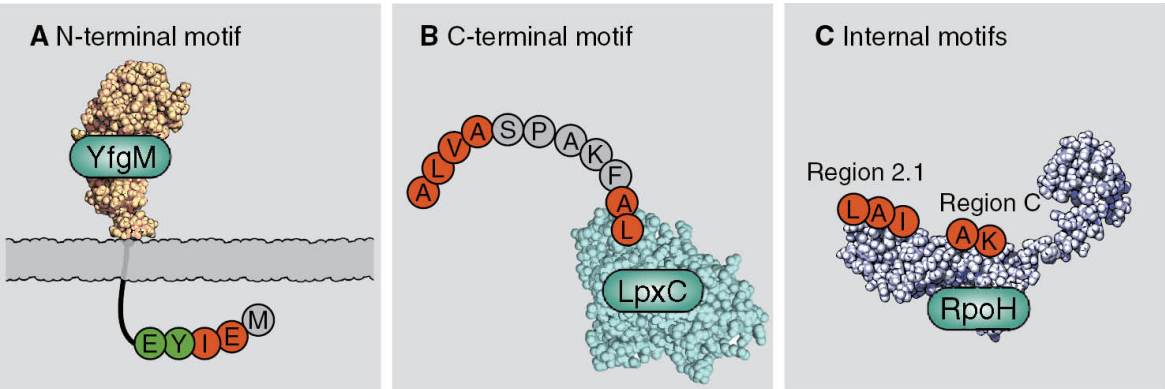


Figure 13. Degron diversity of selected FtsH substrates. The substitution of the amino acids in red leads to the stabilization of the substrates (150). Reprinted from L. M. Bittner *et al.*, 2017, under the terms of the Creative Commons CC BY license.

1.5.6 Hypothesis-independent approaches to identify FtsH substrates and interacting proteins

Most of the substrates known for *E. coli* FtsH have been identified using a trapping approach with catalytically inactive FtsH trapping variants (150, 216, 217). A mutation in the FtsH Walker motifs (Walker^{trap}) inactivates the AAA+ ATPase domain needed for unfolding and translocation of substrates. Another FtsH variant with a mutation in the zinc-binding motif of the protease domain (Protease^{trap}) inactivates the proteolytic site of FtsH (144). Both variants are, therefore, compromised in the degradation of the trapped substrates (Figure 14). The trapped substrates can be crosslinked to FtsH variants and pulled-down via the C- or N-terminal tag of the FtsH variant. The identity of the candidate substrates (and interacting proteins) can be revealed by mass spectrometry. Putative substrates can be confirmed by assessment of their *in-vivo* degradation assay in the presence and absence of the FtsH protease (84, 216, 218).

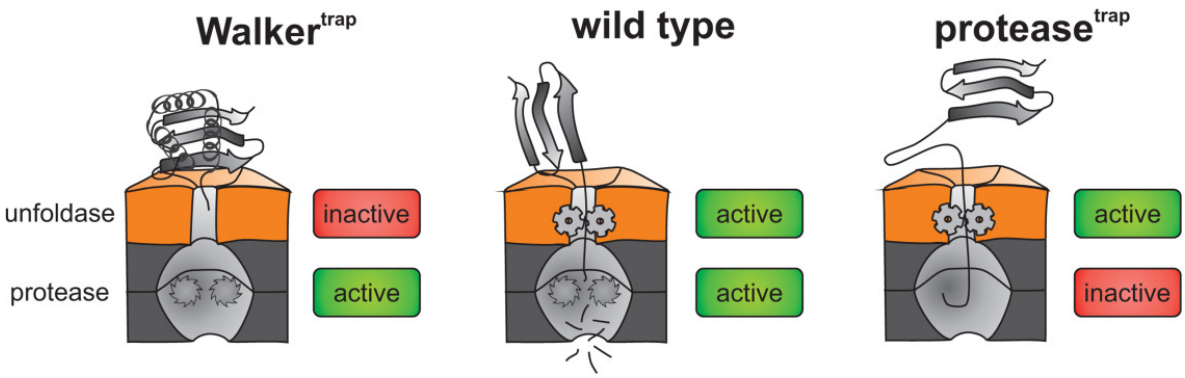


Figure 14. The principle of FtsH trap variants used for substrate identification (144). Reprinted from L. M. Bittner *et al.*, ATP-dependent proteases in bacteria, Biopolymers, 2016, Vol 105, Issue 8, with permission from John Wiley and Sons.

1.6 SIGNIFICANCE

FtsH proteases play an important role in the cellular physiology of different organisms, including contribution to persistence under resource-limited conditions (206, 207). *P. aeruginosa* possesses one FtsH protease, which has not been extensively characterized (207, 208). Unlike most *P. aeruginosa* strains, including PA14 and PAO1, *P. aeruginosa* clone C contains an additional FtsH protease located on the mobile genetic element PACGI-1/TLPQC. Studying FtsH proteases in the worldwide successful *P. aeruginosa* clone C will enhance our understanding of the function of FtsH protease(s) in *P. aeruginosa* physiology and successful adaptation of prevalent strains.

2 AIMS

The thesis aims to investigate genetic fitness determinants encoded on the TLPQC locus. In particular, the role of the protein quality control components, such as the membrane-bound protease FtsH, the stand-alone disaggregase ClpG and their core genome homologs in *P. aeruginosa* clone C strains and *E. coli* strain Fec10.

Paper I – To define the role of the membrane-bound protease FtsH1 encoded on the core genome and its horizontally-acquired xenolog FtsH2 encoded on the clone C specific TLPQC locus in fitness and adaptation of the environmental *P. aeruginosa* clone C strain SG17M

Paper II – To investigate the molecular basis for the differential *in vivo* functionality of FtsH1 and FtsH2 in *P. aeruginosa* clone C member SG17M by genetic and biochemical approaches

Paper III – To study the role of the core genome ClpG disaggregase and the homologous genomic island disaggregase $ClpG_{GI}$ in protein quality control and thermotolerance of *P. aeruginosa* clone C

Paper IV – To study the role of the *dna-shsp20*$_{GI}$*-clpG*$_{GI}$ core thermotolerance operon located on the TLPQC/LHR locus in the human commensal *E. coli* Fec10 isolate and to initially characterize the ST10 type *E. coli* Fec10 strain, a close homolog of *E. coli* K-12

Paper V – To study the frequency of instant double crossover upon homologous recombination of a suicide plasmid designed for gene deletion on the genome of *P. aeruginosa* clone C and other reference strains

3 METHODOLOGICAL CONSIDERATIONS

This chapter provides an overview of the principles and protocols of main laboratory methods that have been applied in this thesis. Detailed protocols can be found in the *Materials and Methods* section of the respective papers.

3.1 CONSTRUCTION OF CHROMOSOMAL DELETION MUTANTS IN *P. AERUGINOSA*

A chromosomal deletion mutant in *P. aeruginosa* can be readily constructed by the replacement of the target open reading frame by an antibiotic resistance cassette (219). This procedure requires the construction of a suicide plasmid with a 'deletion cassette' which consists of FLP-excisable gentamicin (Gm) resistance cassette flanked by >1 kb DNA fragments corresponding to the nucleotide sequences immediately up- and down-stream of the target gene. The deletion cassette was cloned into the suicide plasmid pEX18Tc, which encodes a tetracycline (Tc) resistance cassette as a selectable marker (219) and is transformed into the cloning host *E. coli* Top10. Subsequently, the recombinant suicide plasmid was conjugated into the recipient *P. aeruginosa* strain(s) to replace the target gene by triparental mating with the aid of *E. coli* Top10 harboring the suicide plasmid and *E. coli* HB101 harboring the helper plasmid pRK2013 (Figure 15) (220). Selection on Vogel-Bonner Minimal Medium (VBMM) supplemented with Gm ensures the selection of *P. aeruginosa* conjugates that integrated the suicide plasmid into the chromosome and does not support the growth of the *E. coli* strains (221). Typically, integration of the suicide plasmid into the chromosome occurs through a single crossover recombination event, generating *P. aeruginosa* merodiploid that are Gm^r/Tc^r. A sucrose counterselection step is subsequently required to induce second crossover recombination to excise the suicide plasmid to replace the target gene with a Gm cassette and generate recombinants that are Gm^r/Tc^s.

We observed that *P. aeruginosa* clone C and PA14 strains performed instant double crossover recombination upon plasmid conjugation (findings are described in paper V). Antibiotic sensitivity testing, as well as PCR, were used to distinguish between *P. aeruginosa* recombinants with single and double crossover and to verify the correct deletion mutants (Figure 15). To excise the Gm cassette, the pFLP2 plasmid expressing the FLP recombinase was introduced. The pFLP2 plasmid also bears the *sacB* gene enabling counterselection on sucrose-containing medium and ampicillin (Ap; mediating carbenicillin resistance in *P. aeruginosa*) resistance (219). To subsequently cure the marker-free gene deletion mutant of the pFLP2 plasmid, colonies were grown twice on LB agar supplemented with 5% sucrose and without antibiotics. The excision of the antibiotic cassette was verified by PCR and sensitivity of the colony towards Gm and carbenicillin was confirmed.

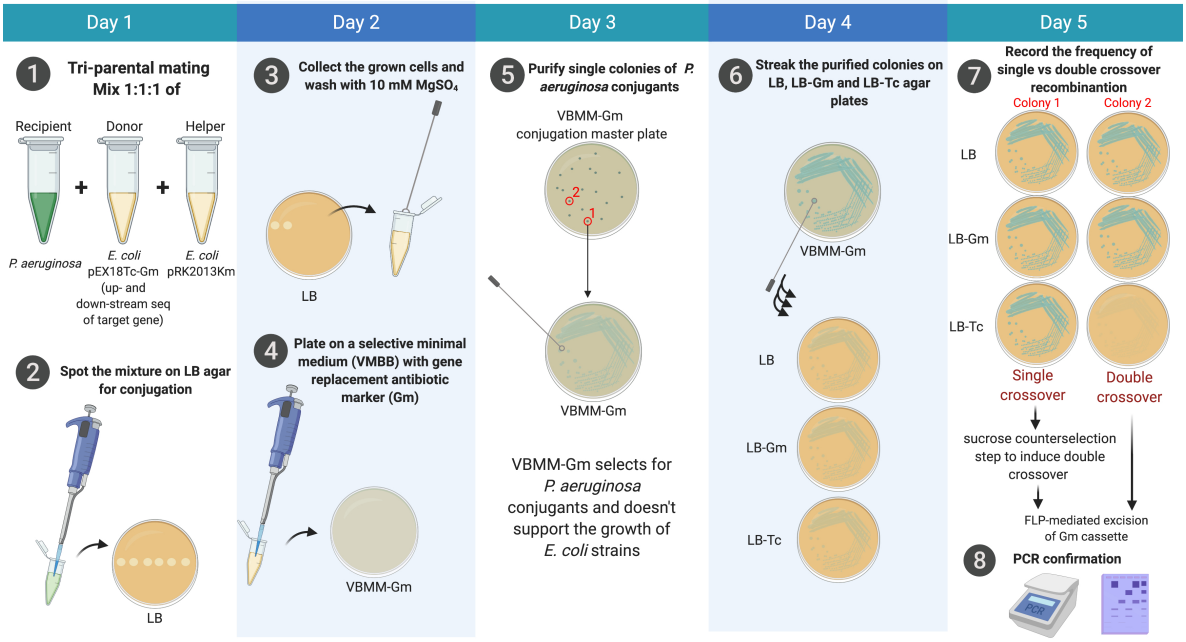


Figure 15. The workflow of the construction of chromosomal deletion mutants in *P. aeruginosa* via homologous recombination and distinguishing recombinants with single versus double crossover recombination events.

3.2 DETECTION OF FTSH1 AND FTSH2 EXPRESSION PATTERN

To determine the relative level of expression of a protein, conventionally Western blot analysis is used for proteins separated according to their molecular weight by SDS-PAGE protein gel electrophoresis. The protein is thereby detected by specific antibodies. To determine the level of expression of FtsH1 and FtsH2 proteins, Myc and 3xFLAG, were introduced into the protein on the C-terminus, respectively, with the respective nucleotide sequences inserted just before the stop codon of the native genes on the chromosome (Figure 16). To construct a tagged gene on the chromosome, the approx. 1 kb DNA fragment containing the sequences upstream of the stop codon of the gene plus the respective tag sequences (including the stop codon) and the 1 kb fragment downstream of the stop codon flanking an FLP-excisable Gm marker were cloned into the suicide vector pEX18Tc. Subsequent conjugal transfer of the recombinant suicide plasmid, selection of conjugants, marker excision and curing, were performed as described for the construction of the chromosomal deletion mentioned above. The native protein production of FtsH1 and FtsH2 was tested in LB and M63 minimal medium at 37 ºC and 20 ºC in logarithmic, stationary and late stationary phases. Using a proven tag for protein detection has advantages, such as the commercial availability of well-characterized antibodies, high specificity and applicability to different proteins. On the other hand, the tag may interfere with the protein structure, stability and function. Indeed, we observed slight growth retardation of the cells producing FtsH1 with the Myc-tag at the C-terminal end (84).

3.3 FORMATION OF HETERO-OLIGOMERS BY FTSH1 AND FTSH2

Hetero-oligomerization of different FtsH homologs has been observed in cyanobacteria, which can lead to novel functionality (222, 223). Homo- and hetero-oligomerization between FtsH1 and FtsH2 were tested by constructing open reading frames encoding bait proteins of wild type FtsH1-Strep and FtsH2-Strep, which were cloned into the L-arabinose-inducible pJN105 vector. The bait proteins were introduced into *P. aeruginosa* SG17M strain containing *ftsH1-myc* at the native chromosomal locus (Figure 16A) and vice versa, the SG17M strain with *ftsH2-3xFLAG* on the chromosome (Figure 16B). Bait protein expression was induced by 0.05% L-arabinose at OD_{600} = 0.4 and cells were harvested around 2 h post-induction at OD_{600} = 0.8. Proteins were crosslinked by adding 1% formaldehyde and incubated for 10 min. Afterward, the crosslinker was quenched with 1 M glycine and the cells were lysed by sonication. Subsequently, the bait protein was purified using the Strep tag, which leads to stable co-purification of the associated proteins. Crosslinking was reversed by boiling for 20 min and the co-purified chromosomally encoded Myc and 3xFLAG tagged proteins were detected by immunoblotting.

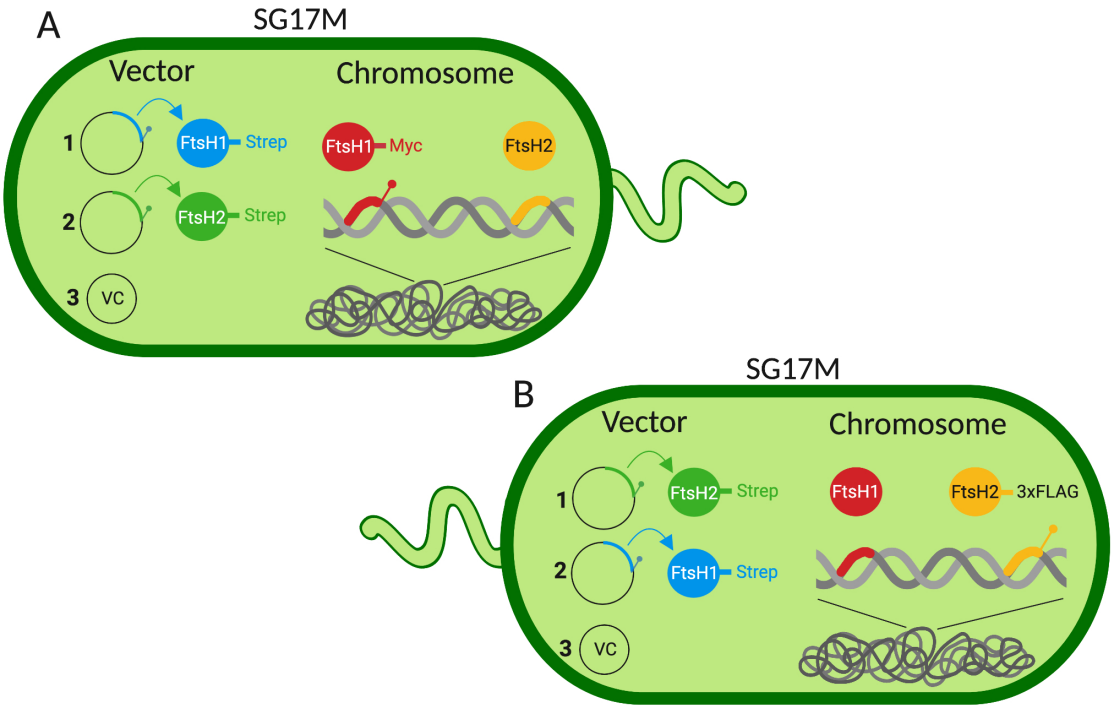


Figure 16. Schematic representation of the experimental strategy to assess FtsH1/FtsH2 protein-protein interactions. Bait proteins FtsH1-Strep and FtsH2-Strep cloned into an expression vector were expressed in *P. aeruginosa* SG17M encoding (A) chromosomal *ftsH1-myc* or (B) chromosomal *ftsH2-3xFLAG*.

3.4 THE TRAP-BASED SUBSTRATE IDENTIFICATION APPROACH

Substrate identification of the FtsH protease has been mainly performed in *E. coli* (144, 216, 224) but also in cyanobacteria (225, 226). To identify novel substrates for the FtsH1 and FtsH2 proteases in *P. aeruginosa* SG17M, a variant of the above-described pull-down assay was applied. In this case, the trap proteins ($FtsH^{trap}$) $FtsH1^{trap}$ and $FtsH2^{trap}$ with an amino acid exchange in the first histidine in the conserved zinc-binding motif HEXXH, H416Y and H429Y, respectively, were constructed to serve as bait (Figure 17). Thereby, the protease activity of the $FtsH^{trap}$ variant proteins is compromised, while substrate binding, ATP

hydrolysis and subsequent substrate unfolding and translocation are retained (Figure 17) ((216, 218)). The principle of trap proteins, consisting of a catalytically inactivated protease domain while maintaining the AAA+ ATPase domain activity, has been previously applied for Lon and ClpXP (227, 228). The trap variant proteins with a C-terminal Strep tag cloned in the pJN105 plasmid were induced with 0.05% L-arabinose in the respective *ftsH* single deletion mutant background of *P. aeruginosa* SG17M. Cells were harvested at OD_{600} = 1 followed by crosslinking, quenching, cell lysis, purification of variant proteins, reversal of crosslinking and separation and analysis by SDS-PAGE gels as described above. Distinct candidate protein bands were selectively and reproducibly co-purified with the trap proteins, then cut from the SDS-PAGE gel, and identified by mass spectrometry. Cells harboring empty vectors were used as the Streptavidin resin control, while gel bands of crosslinked lysate that were not reversed, were used to identify potential transient protein candidates. Candidate substrates were verified by *in vivo* degradation in the wild type and *ftsH* single and double deletion strains.

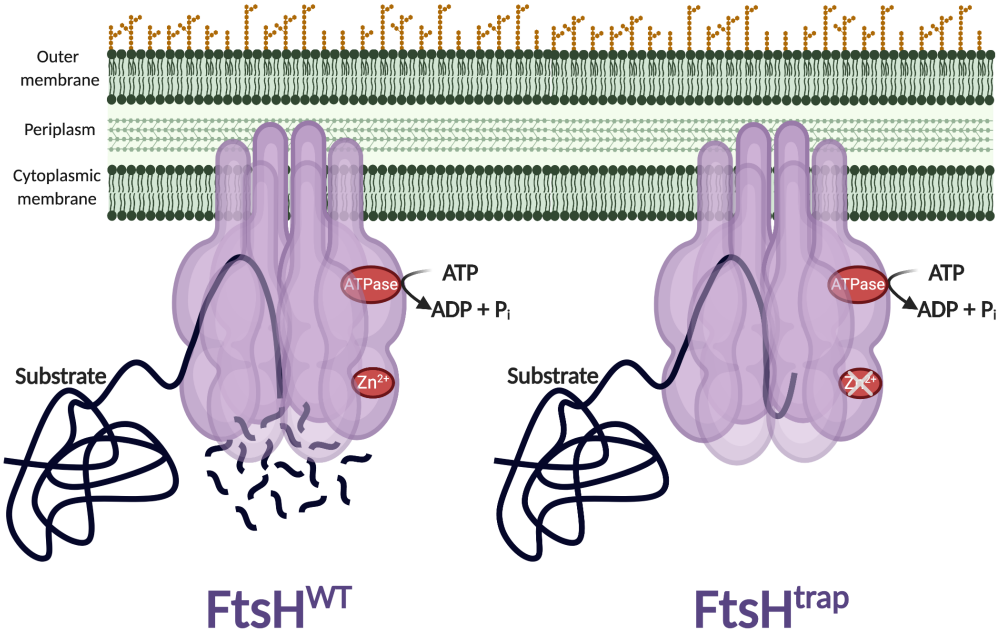


Figure 17. Principle of the trapping approach for the AAA+ protease FtsH. The active AAA+ ATPase domain hydrolyzes ATP to unfold and translocate the substrate into the proteolytic chamber. The substrate is trapped by the FtsH^{trap} construct (shown on the right), where the zinc ion can no longer bind to the mutated zinc-binding motif of the protease domain, resulting in disruption/termination of the proteolytic activity. In contrast, the substrate is degraded by FtsH^{WT} (shown on the left), where zinc can bind to the intact zinc-binding motif.

The substrate trapping approach used for FtsH was validated by the co-purification of known FtsH substrate and modulator proteins. However, the experimental method used has some limitations. For example, using a 1D protein gel to identify the substrates results in a low resolution and potentially restricts the identification of the less abundant proteins. A methodological extension leading to higher resolution and numbers of identified proteins would be to use a gel-free approach by LC-MS/MS. Furthermore, the identified substrates reflect the physiological function of FtsH at only the specific growth stage when the substrates were crosslinked so that the trapped substrates are a fraction of the substrates of FtsH over the entire growth phase. To address this issue, alternative growth phases and conditions can be used.

3.5 FTSH PURIFICATION

FtsH protein purification was performed as previously described (229, 230), with some modifications summarized in Figure 18. Overexpression of membrane proteins is often troublesome since they compromise the membrane integrity. As a result, the overexpression of FtsH was found to be deleterious in *E. coli* (231). The FtsH purification protocol used in this thesis is based on the extraction of FtsH from inclusion bodies collected from the insoluble fraction of the cell lysate. To solubilize the inclusion bodies, the ionic detergent N-lauroylsarcosine was used in the presence of ATP. Upon dissolution of the inclusion bodies, the transmembrane segments of FtsH are inserted into the detergent micelles (229). Subsequent refolding steps occur when the ATP binding domain refolds around the Mg^{2+} and ATP during the process of gradual dilution of N-lauroylsarcosine micelles in NP-40 (Igepal) on the purification column. The presence of ATP during solubilization and refolding helps FtsH assemble into higher-order structures and increases the proportion of active FtsH molecules (229).

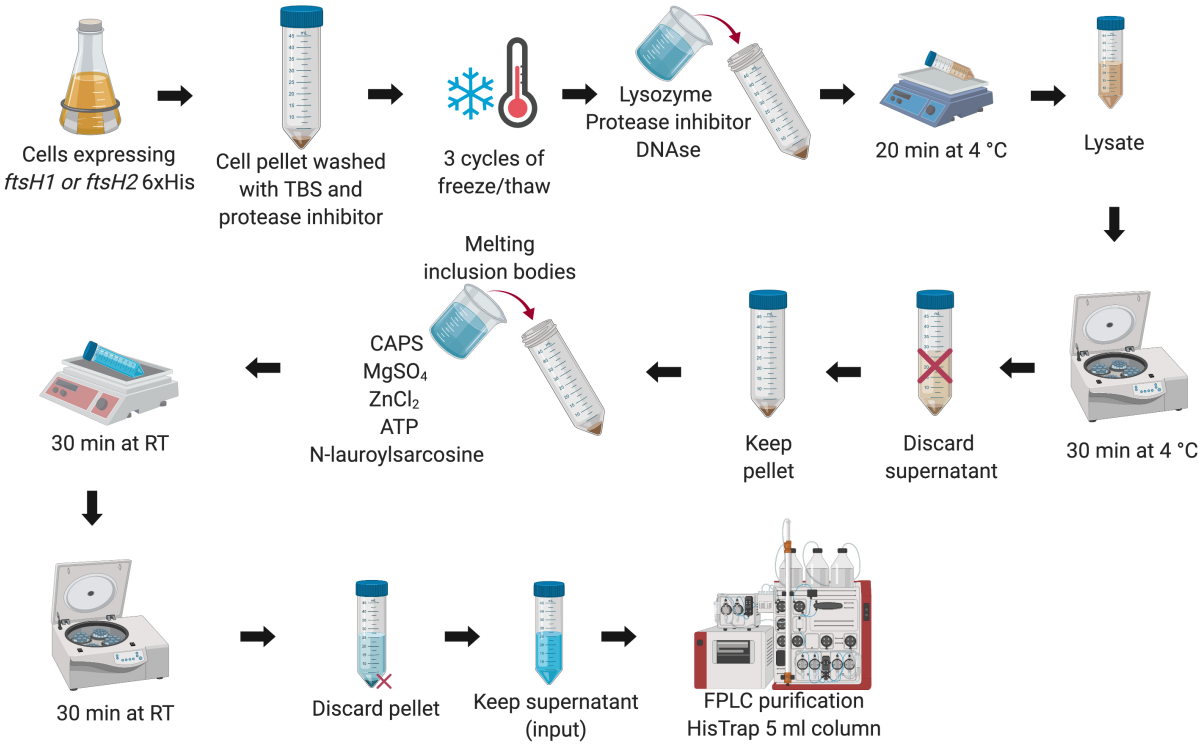


Figure 18. The workflow of FtsH solubilization and refolding steps from the inclusion bodies optimized for *P. aeruginosa* in collaboration with Tania A. Baker and Robert T. Sauer laboratories.

4 RESULTS AND DISCUSSION

4.1 PAPER I: TWO FTSH PROTEASES CONTRIBUTE TO FITNESS AND ADAPTATION OF *PSEUDOMONAS AERUGINOSA* CLONE C STRAINS

In **Paper I**, we aimed to identify the role of the ATP-dependent membrane protease FtsH of *P. aeruginosa* environmental isolate SG17M encoded on the core genome by *ftsH1* and on the PACGI-1/TLPQC genomic island by its homolog *ftsH2*. The deletion of the core genome copy *ftsH1* showed a pleiotropic effect on a broad range of fitness traits such as growth, flagellar and type IV pili motility, biofilm formation under steady-state and continuous flow conditions, antibiotic resistance, secretion and production of secondary metabolites, heat and oxidative stress tolerance. While deletion of the genomic island copy *ftsH2* in the wild-type background hardly manifested an effect, the *ftsH1* and *ftsH2* double deletion mutant displayed an additive effect on most of the phenotypes tested compared to the *ftsH1* single deletion mutant. These findings indicated a backup functionality of *ftsH2* in the absence of *ftsH1*. In congruence with the mutant phenotypes, phenotypes fully complemented by *ftsH1* were only partially complemented by *ftsH2*. Therefore, FtsH1 and FtsH2 – despite their highly conserved enzymatic AAA+ ATPase and protease domains – contribute unequally to phenotypes linked to fitness and adaptation of SG17M (Figure 19). Similar to *P. aeruginosa*, the deletion of *ftsH* in *Bacillus subtilis* results in a pleiotropic effect, including retardation of growth (161). In addition to the pleiotropic effect of *ftsH* deletion, *ftsH* was shown to be crucial for general fitness in *P. aeruginosa* PA14 during growth-arrest caused by energy limitation (206).

The heat shock response needs to be tightly controlled to avoid deleterious activation of, e.g., heat-inducible proteases. FtsH rapidly degrades the heat-shock transcription factor RpoH (σ^{32}) at the permissible growth temperature in *E. coli* (194-196). In the wild-type *P. aeruginosa* clone C strain SG17M and the Δ*ftsH2* background, σ^{32} induced from an expression vector was degraded rapidly in less than 10 min post-induction at 37 ºC. On the other hand, σ^{32} was degraded in the Δ*ftsH1* and Δ*ftsH1*Δ*ftsH2* backgrounds after 30 and 60 min post-induction, respectively. Moreover, the steady-state levels of σ^{32} upon induction were approximately >30 times higher in the Δ*ftsH1* and Δ*ftsH1*Δ*ftsH2* background compared to the wild-type and Δ*ftsH2* background. Therefore, σ^{32} is kept under a tight control mainly by *ftsH1* and to a minor extent by *ftsH2* in *P. aeruginosa* SG17M.

In *E. coli,* FtsH is essential for viability as it maintains the balance of LPS/phospholipid in the outer membrane. FtsH achieves this by degrading the LPS biosynthesis enzymes, such as the key enzymes LpxC that synthesizes the hydrophobic anchor of LPS called lipid A and KdtA that catalyzes subsequent reactions (160, 192, 193). Of note, shifting the temperature-sensitive *ftsH* in *E. coli* to the non-permissible temperature of 42 ºC caused abnormal production of new membranes located in the periplasm probably due to over-accumulation of LPS (160). Similarly, depletion of *ftsH* in the Lyme disease pathogen *Borrelia burgdorferi* leads to the formation of large membrane distortions (232). However, LpxC degradation in *P. aeruginosa* is not FtsH-dependent (233); therefore, FtsH is required for optimal growth but not being essential for viability (206-208, 234). Indeed, no membrane abnormalities were found in the *ftsH* deletion mutants grown in standard LB medium at 37 ºC in *P. aeruginosa* SG17M. Additionally, other parameters such as cell morphology are not altered, as the cell length of *ftsH*-depleted *P. aeruginosa* PA14 does not differ from the wild-type in the

exponential phase of growth in LB medium at 37 ºC (207). Therefore, proteases other than FtsH maintain this vital step in membrane homeostasis in *P. aeruginosa*.

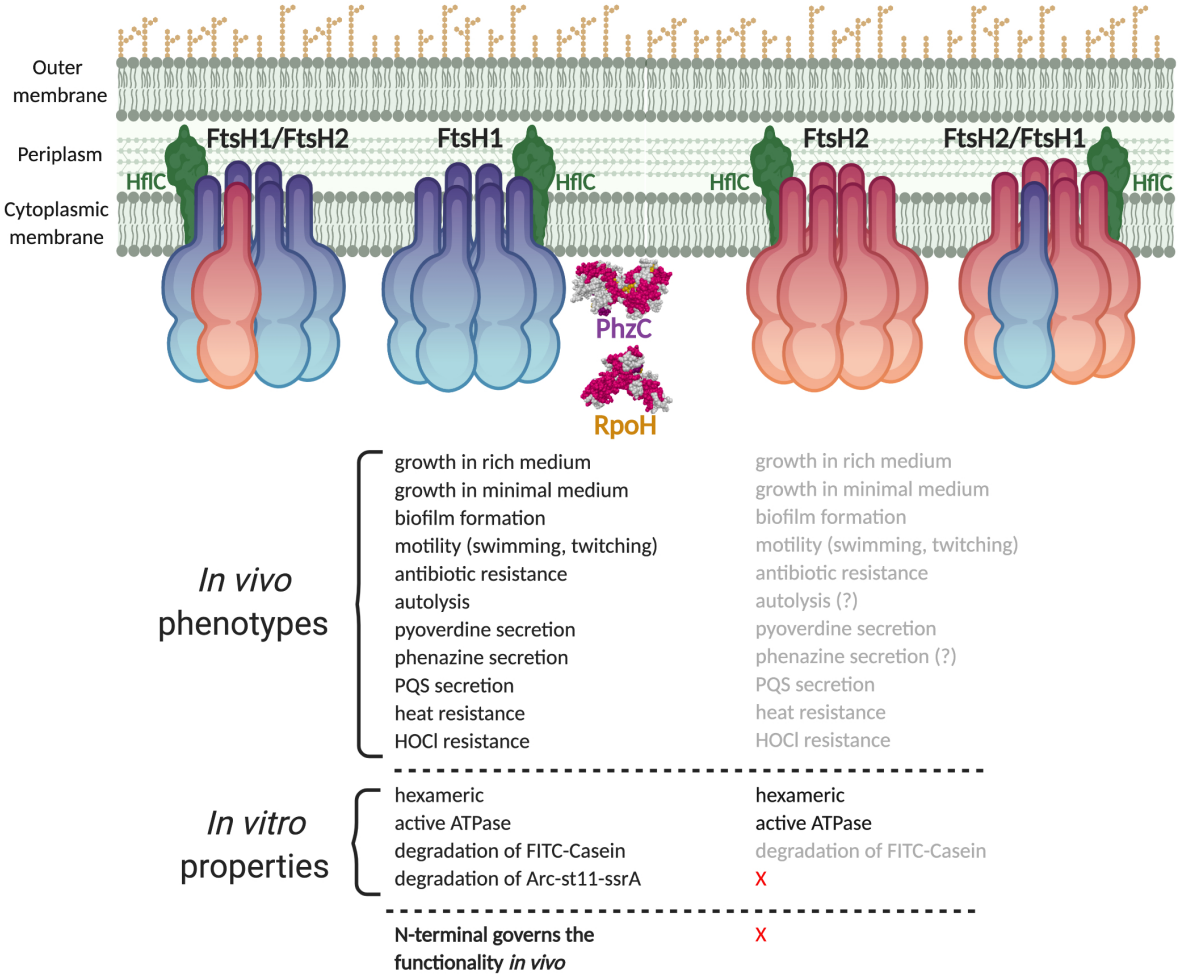


Figure 19. Schematic summary representing the *in vivo* functionalities and *in vitro* properties of FtsH1 and FtsH2 in *P. aeruginosa* SG17M. Phenotypes affected by FtsH1 are listed in black, while phenotypes that are backed up by FtsH2 in the absence of FtsH1 are listed in grey. The homo- and hetero-oligomeric complexes of FtsH1 and FtsH2 are represented in different colors for the monomers. The modulator protein HflC associated with FtsH1 and FtsH2 is indicated in green. The substrates mainly degraded by FtsH1 are shown.

Since FtsH2 showed a weak contribution to the tested phenotypes, we wondered if the protein is produced at all. Strikingly, the chromosomally encoded FtsH2-3xFLAG was constitutively produced throughout logarithmic, stationary and late stationary phases of growth in a rich and minimal medium at the environmental temperature of 20 ºC and human body temperature of 37 ºC. On the other hand, the chromosomally encoded FtsH1-Myc was nearly absent in the late stationary phase of growth. Similar to the production pattern of FtsH2, other neighboring TLPQC genes products involved in proteostasis were predominantly expressed in the late stationary phase, such as $sHsp20_{GI}$ and $ClpG_{GI}$ (74, 138). FtsH particularly supports cell viability in *P. aeruginosa* PA14 entering a state of growth arrest, e.g., upon entering the stationary phase in LB medium or the logarithmic phase grown in a medium lacking organic carbon (207). Therefore, we speculate that FtsH2 provides a distinct fitness advantage in the late stationary phase, where FtsH1 is absent.

The presence of four FtsH proteases in the cyanobacterium *Synechococcus* promotes the formation of hetero-oligomers with distinct functionalities compared to the homo-oligomers (209, 222, 223). In *E. coli,* FtsH protease monomers assemble into homo-hexamers in the inner cell membrane (165-169). To investigate whether FtsH1 and FtsH2 of *P. aeruginosa* SG17M interact *in vivo*, *ftsH1-Strep* and *ftsH2-Strep* cloned into an expression vector were introduced into strains with the reciprocal protein-tagged on the chromosome. Subsequent crosslinking and pull-down using the Strep-tagged protein as a bait followed by immunoblot detection of the proteins with the respective tag-specific antibodies identified FtsH1 and FtsH2 to form homo- and hetero-oligomers. Consequently, compared to most *P. aeruginosa* clones that encode one copy of the FtsH protease, the horizontally acquired FtsH2 might widen the functionality of the core genome protease FtsH1 through hetero-oligomerization and even FtsH2 homo-oligomerization (Figure 19).

In *E. coli*, *ftsH* is a pleiotropic gene that translates into a protein with a diverse substrate pool (150). To identify substrates for FtsH in *P. aeruginosa* SG17M, Strep tagged trap proteins that are deficient in proteolytic activity, but proficient in ATPase activity, were generated for FtsH1 and FtsH2 cloned into an expression vector (216). The trap variant proteins were then used as baits to trap substrates by crosslinking, followed by Strep tag mediated pull-down. The identity of the potential substrates co-purified with the variant proteins was determined by mass spectrometry. The FtsH modulator protein HflC (180) and ATP synthase subunit alpha, known to be a modulator and a substrate in *E. coli*, respectively, (184, 235) were pulled-down with FtsH1 and FtsH2 thus validating the methodology. In addition, LepB that codes for the Signal peptidase I protein to collaborate with FtsH for processing and translocation of colicin D across the membrane in *E. coli* (188-190), was pulled-down specifically with FtsH2.

On the other hand, a potential substrate that was pulled-down with FtsH1 was HslU, the ATP binding subunit of the AAA+ protease HslUV. A mutation in *hslU* in the absence of *ftsH* in *P. aeruginosa* PA14 affected fitness negatively, indicating that *hslU* and *ftsH* have growth-aggravating interaction. This proposes the possibility that FtsH, together with other proteases, degrades protein aggregates resulting from cell aging and growth arrest (207).

Lastly, the phenazine biosynthesis protein PhzC was confirmed to be a substrate of FtsH1 by the above described *in vivo* degradation assay. The PhzC production in the Δ*ftsH1* and Δ*ftsH1*Δ*ftsH2* backgrounds was consistent with the over-accumulation of pyocyanin pigment. Although the number of proteins analyzed was limited, the presence of distinct substrates pulled-down with FtsH1 and FtsH2 in *P. aeruginosa* SG17M suggests a yet-to-be-discovered, unique functionality of each protein. In addition, FtsH limited the secretion of the green fluorescent siderophore (pyoverdines) and stimulated the secretion of PQS signaling molecules. Therefore, mainly FtsH1 and partially FtsH2 act as a major regulator for secondary metabolite production and/or secretion in *P. aeruginosa* SG17M (Figure 20).

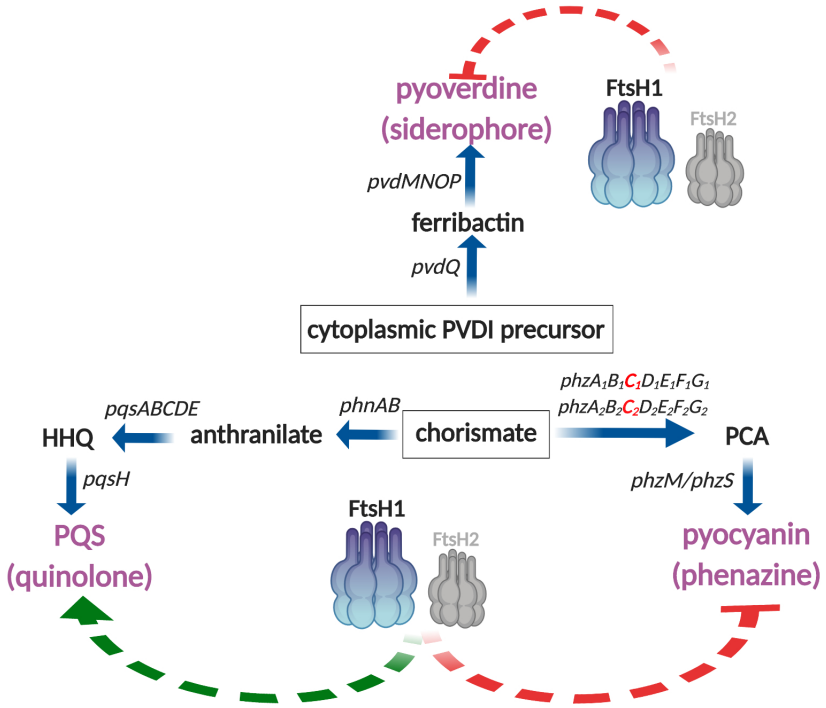


Figure 20. FtsH proteases are involved in the regulation of secondary metabolites in *P. aeruginosa* SG17M.

4.2 PAPER II: THE N-TERMINUS OF FTSH1 BUT NOT FTSH2 IS REQUIRED FOR NORMAL GROWTH OF *P. AERUGINOSA* CLONE C STRAINS

In **paper II**, we investigated the underlying molecular basis for the *in vivo* differential activity of FtsH1 (*Pa*FtsH1) and FtsH2 (*Pa*FtsH2) in *P. aeruginosa* SG17M observed in **Paper I** through biochemical and genetic approaches. To test the functionality of *Pa*FtsH1 and *Pa*FtsH2 *in vitro*, the proteins were first purified with a 6xHis tag upon overexpression from an expression vector along with the well-studied FtsH from *E. coli* (*Ec*FtsH). Size exclusion column chromatography showed that the native purified *Pa*FtsH1 and *Pa*FtsH2 are assembled into hexameric homo-oligomers. Purified *Ec*FtsH, *Pa*FtsH1 and *Pa*FtsH2 showed comparable ATP hydrolysis rates *in vitro*, but differential degradation for the model substrates FITC-casein and Arc-st11-ssrA.

As observed in **paper I**, wild-type *ftsH1* fully complemented the growth retardation phenotype of SG17M Δ*ftsH1*Δ*ftH2*, whereas wild-type *ftsH2* partially complement the growth retardation phenotype. *Pa*FtsH1 and *Pa*FtsH2 share highly conserved AAA+ ATPase and protease domains flanked by less conserved N- and C-terminal regions. The N-terminal domain swaps between *Pa*FtsH1 and *Pa*FtsH2 surprisingly showed that the differential *in vivo* complementation activity was governed by the first N-terminal 151 and 154 amino acids of *Pa*FtsH1 and *Pa*FtsH2, respectively, which were identified as the most divergent regions in FtsH.

The swapped N-terminal regions of FtsH1 and FtsH2 consisted of two transmembrane helices flanked by a periplasmic domain and followed by a short cytoplasmic linker upstream of the AAA+ module. In order to identify the subdomain(s) of the N-terminal region that dictated the differential *in vivo* activity, we constructed additional hybrid proteins with an iterative shorter N-terminal domain. As a result, the short glycine-rich cytoplasmic linker connecting

the N-terminal region with the AAA+ module and the periplasmic domain were shown to be major positive determinants of FtsH1 *in vivo* activity.

The ATPase and proteolytic activity of FtsH require membrane integration and homo-oligomerization driven by the N-terminal region (176, 177). In addition, the periplasmic domain of FtsH allows interaction with the periplasmic modulator proteins HflK/HflC (166, 180), that negatively regulate FtsH proteolysis towards some substrates like SecY (184, 197). The fact that *Pa*FtsH2 is an active ATPase that forms homo-oligomers shows that the N-terminal sequence of FtsH2 does not impair functionality per se through hindering homo-oligomerization or inhibiting ATPase activity. Besides, the periplasmic domain of FtsH2 seems to be capable of binding major regulatory proteins, since FtsH2, as well as FtsH1, were found to bind HflC *in vivo* (**paper I**). As FtsH often fine-tunes its substrate specificity and catalytic activity through modulator/escorting partner proteins (Table 1), it is tempting to speculate that, as the analysis of interacting proteins has been limited, there are yet-to-be-discovered modulator proteins in *P. aeruginosa* that – through interacting with the N-terminal part – promote substrate selectivity and degradation activity.

4.3 PAPER III: STAND-ALONE CLPG DISAGGREGASE CONFERS SUPERIOR HEAT TOLERANCE TO BACTERIA

In **paper III**, we studied the role of the core genome AAA+ ClpG disaggregase and the homologous TLPQC genomic island disaggregase $ClpG_{GI}$ in protein quality control and thermotolerance of *P. aeruginosa* clone C. The classical AAA+ Hsp100 chaperone ClpB requires the partner co-chaperone system DnaKJE that binds and escorts protein aggregates to ClpB and concurrently stimulates the ATPase activity of ClpB (123, 135, 136). On the other hand, ClpG/$ClpG_{GI}$ was found to bind cellular aggregates independently of partner proteins because of the unique N-terminal extension of ClpG/$ClpG_{GI}$. Also, the stand-alone ClpG/$ClpG_{GI}$ disaggregases can replace the function of the classical bi-chaperone system due to its high intrinsic AAA+ ATPase activity, which enables potent disaggregation activity. Therefore, the unique features of ClpG/$ClpG_{GI}$ as a disaggregase provide superior lethal heat tolerance to *P. aeruginosa* clone C SG17M.

We were interested in identifying native substrates for the disaggregase $ClpG_{GI}$ in *P. aeruginosa* SG17M (unpublished data). A $ClpG_{GI}^{trap}$ protein was constructed by mutating the Walker B motif in both AAA+ domains and cloning the Strep tagged protein variants in the pJN105 expression vector. The variant trap proteins $ClpG_{GI\text{-}E383A/E723A}$ were produced in the triple mutant *P. aeruginosa* SG17M Δ*clpB*Δ*clpG*Δ*clpG*$_{GI}$. Subsequent crosslinking, quenching, lysis, pull-down and mass spectrometry analysis were performed. The resin and crosslinking controls are indicated in Figure 21 and the identity of the candidate substrates is listed in Table 2. Although the potential substrates were not further confirmed, the pull-down of ribosomal proteins may indicate that $ClpG_{GI}$ is either interacting tightly with ribosomes and subsequently involved in folding newly synthesized polypeptides and/or maintaining the quality of ribosomal proteins, extending its role beyond disaggregase functionality for thermally aggregated proteins.

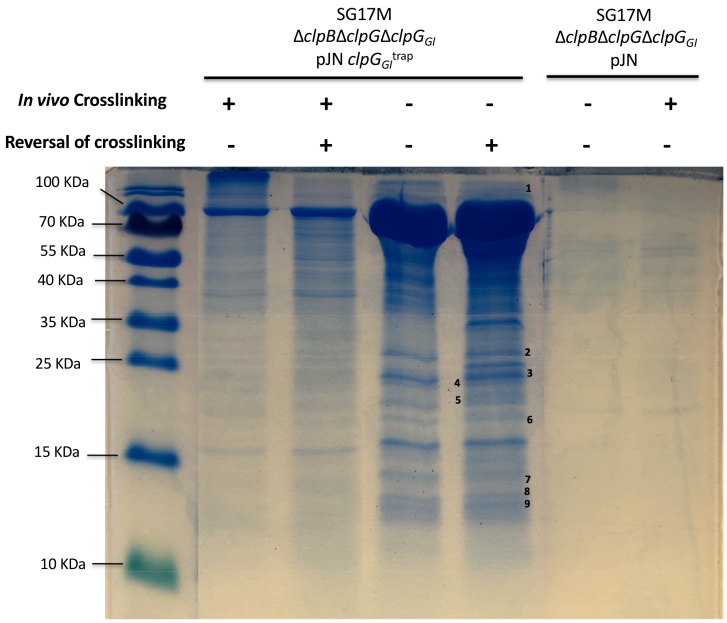


Figure 21. Results of the pull-down of potential substrates for $ClpG_{GI}$. Crosslinked and non-crosslinked strain *P. aeruginosa* SG17M Δ*clpB*Δ*clpG*Δ*clpG*$_{GI}$ *pclpG*$_{GI}^{trap}$ and crosslinked bead control with an empty vector were loaded onto an SDS-PAGE gel (4% stacking and 16% separating gel). To reverse crosslinking, samples were boiled for 20 min. The nine potential substrates co-purified with $ClpG_{GI}^{trap}$ are numbered.

Table 2. Potential substrates for *P. aeruginosa* $ClpG_{GI}$

Band no.	Protein name
1	DNA-directed RNA polymerase subunit beta
2	30S ribosomal protein S2
3	30S ribosomal protein S3
4	30S ribosomal protein S4
5	ABC transporter substrate-binding protein
6	50S ribosomal protein L5
7	Tyrosine recombinase XerD/XerC
8	50S ribosomal protein L14
9	50S ribosomal protein L19

4.4 PAPER IV: A RECENTLY ISOLATED HUMAN COMMENSAL *ESCHERICHIA COLI* ST10 CLONE MEMBER MEDIATES THERMOTOLERANCE ON A P1 PHAGE DERIVED INCY PLASMID

In **paper IV**, we initially characterized a modern commensal *E. coli* strain called Fec10 isolated from the gastrointestinal tract of a healthy individual. Fec10 is a member of the ST10 clonal type closely related to *E. coli* K12, which harbors a 155.9 kb plasmid (termed pFec10) containing TLPQC/LHR encoded *dna-shsp20*$_{GI}$-*clpG*$_{GI}$. Since this heat locus, particularly *clpG*$_{GI}$, was associated with the elevated heat tolerance in *P. aeruginosa* clone C **(paper III)**, we were wondering to which extent *clpG*$_{GI}$ and the tri-operon *dna-shsp20*$_{GI}$-*clpG*$_{GI}$ contribute to lethal heat tolerance of the highly tolerant commensal *E. coli* Fec10 strain.

The deletion of *clpG*$_{GI}$ and tri-operon *dna-shsp20*$_{GI}$-*clpG*$_{GI}$ in *E. coli* Fec10 severely reduced the heat tolerance upon exposure to 60 °C for 15 min compared to the wild-type, which was

almost not affected. The heat sensitivity of the deletion mutants was partially restored by the provision of *clpG$_{GI}$* and *dna-shsp20$_{GI}$-clpG$_{GI}$* encoded on the expression vector pBAD30. In addition, the heat sensitivity of *E. coli* MC4100 Δ*dnaK* at 50 °C was fully complimented by *clpG$_{GI\text{-}Fec10}$* and the highly homologous *clpG* from *P. aeruginosa* SG17M (*clpG$_{GI\text{-}SG17M}$*). Also, *clpG$_{GI\text{-}Fec10}$* and *clpG$_{GI\text{-}SG17M}$* provided improved heat tolerance to *E. coli* MC4100 Δ*clpB* compared to the strain with a plasmid expressing *clpB.* This indicates that ClpG$_{GI}$ can compensate the functionality of the classical bi-chaperone system ClpB/DnaK in *E. coli.* Therefore, the presence of the horizontally transferred heat locus, in addition to the ClpB/DnaK bi-chaperone system, provides extreme thermotolerance to the commensal strain Fec10.

We screened a panel of gastrointestinal commensal isolates for the presence of the *shsp20$_{GI}$* gene as a marker for the occurrence of the TLPQC/LHR locus. Four out of 32 tested isolates harbored the *shsp20$_{GI}$* gene, three of which were thermoresistant upon exposure to 55 °C for 45 min compared to other *shp20* negative strains. In order to test the limit of the thermotolerance phenotype provided by the heat locus in Fec10, an extremely elevated temperature of 65 °C was applied for 5 min, for which the Fec10 isolate showed less than ten-fold reduction of viability. Raw food processes that include mild to moderate temperature upshift put a selective pressure that can select for thermoresistant bacteria. Nonetheless, the locus of heat tolerance was found to be acquired by clinical isolates, such as the multi-drug resistance *Klebsiella pneumoniae* C132-98 (81) and extended-spectrum β-lactamase (ESBL)-producing *E. coli* C604-10 (116).

Similar to the results from our biochemical characterization of ClpG$_{GI\text{-}SG17M}$ in **paper III**, the purified ClpG$_{GI\text{-}Fec10}$ had a high basal ATPase activity without substrate stimulation, unlike the ATPase activity of ClpB that was stimulated upon interacting with the casein-loaded DnaKJE system. Consistent with *P. aeruginosa* ClpG/ClpG$_{GI}$, ClpG$_{GI\text{-}Fec10}$ was able to disaggregate two heat-denatured standard substrates, malate dehydrogenase (MDH) and luciferase independently without the help from partner proteins. The ClpB co-chaperone partner proteins (DnaKJE) significantly inhibited ClpG$_{GI\text{-}Fec10}$ disaggregation activity since both ClpG$_{GI\text{-}Fec10}$ and DnaKJE competed for substrate binding. Although binding of aggregates to the N-terminal extension of ClpG$_{GI\text{-}Fec10}$ was not experimentally determined, we hypothesize it behaves similarly to ClpG$_{GI\text{-}SG17M}$, due to the high sequence homology and the observed stand-alone substrate disaggregation activity of ClpG$_{GI\text{-}Fec10}$.

We were wondering how exposure to elevated temperatures would affect the thermodynamic stability of ClpG$_{GI}$ compared to ClpB. We, therefore, tested the change in the secondary structure of the disaggregases upon gradual temperature upshift by Circular Dichroism (CD) in the presence and absence of Mg^{2+}/ATPγS. The melting temperature of the secondary structure of ClpB in the absence of Mg^{2+}/ATPγS was 53.1 °C compared to 62.9 °C and 64.5 °C of ClpG$_{GI\text{-}SG17M}$ and ClpG$_{GI\text{-}Fec10}$, respectively. In the presence of Mg^{2+}/ATPγS, the melting temperature of ClpB was 61.8 °C compared to 69.7 °C and 68.9 °C of ClpG$_{GI\text{-}SG17M}$ and ClpG$_{GI\text{-}Fec10}$, respectively. Therefore, ClpG$_{GI}$ can retain its secondary structure, and consequently function, at a higher temperature compared to ClpB. Besides the inherent biochemical properties that make ClpG/ClpG$_{GI}$ a superior disaggregase in *P. aeruginosa* and *E. coli*, ClpG was more robust in solubilizing tighter aggregates formed at higher temperatures *in vitro* and *in vivo* compared to ClpB (139).

4.5 PAPER V: HIGH FREQUENCY OF DOUBLE CROSSOVER RECOMBINATION FACILITATES GENOME ENGINEERING IN *PSEUDOMONAS AERUGINOSA* PA14 AND CLONE C STRAINS

In **paper V,** we report on instant double crossover observed in *P. aeruginosa* upon homologous recombination of a suicide plasmid designed for gene deletion. A two-step allelic exchange of a target gene with an antibiotic marker introduced on a suicide vector via homologous recombination is a frequently used method to manipulate the genome of *P. aeruginosa* (74, 219). Conventionally, the suicide vector with homologous up- and down-stream sequences of the target gene flanking an antibiotic cassette, integrates via a single cross over into the chromosome. A second homologous recombination via a counterselection step to excise the suicide plasmid backbone replaces the target gene with an antibiotic resistance marker. In order to systematically investigate the single and double crossover frequencies of a suicide plasmid in *P. aeruginosa*, we tested the crossover frequencies upon deletion of four genes (*clpB*, *clpG*, *ibpA* and *exsA*) in five different *P. aeruginosa* strains; three belonging to clone C strains (SG17M, SG31M, 8277) and two belonging to well-studied non-clone C reference strains (PAO1 and PA14).

In the limited panel of four target genes, more than 1000 colonies were assessed for single and double crossover recombination by testing antibiotic sensitivity and PCR using primers flanking the homologous recombination region. We observed that the frequency of the double crossover was, on average, >70% in PA14 and the three clone C strains tested. In contrast, the double crossover frequency was, on average, less than 20% in the PAO1 strain. The up- and down-stream sequences used for homologous recombination of the target genes *clpB*, *clpG*, *ibpA* and *exsA* were almost 98% identical between PA14 and PAO1. Therefore, the differences in double crossover frequencies between these two strains were not dependent on the nucleotide sequences flanking the target genes. From a practical standpoint, upon the availability of the gene replacement vector, replacement of an open reading frame with an antibiotic marker in clone C strains and PA14 can take less than six days, which facilitates the genome engineering of these strains by skipping the counter selection step (Figure 15). Clone C isolates and PA14 belong to the most common clonal group of *P. aeruginosa*, unlike PAO1 that belongs to an uncommon clonal group (236). Consequently, we assume that the ability to facilitate homologous recombination can be indirectly linked to the enhanced ability for transmission and persistence of abundant clonal strains.

In summary, we demonstrated that the survival advantage of the clone C specific genomic island PACGI-1/TLPQC is mainly provided through backup functionality of various phenotypes by FtsH2 (**paper I)** and mediation of a dominant superior heat tolerance phenotype by $ClpG_{GI}$ **(paper III)**. In **paper V**, we documented an instant double crossover event for clone C strains and the PA14 representative suggesting superior recombination activity, which might be an additional hallmark of successful clones such as clone C and clone PA14.

5 CONCLUSIONS

This thesis investigates the molecular basis of fitness advantages of *P. aeruginosa* SG17M that belongs to the worldwide prevalent clone C strain cluster found in clinical and environmental settings. Gene products encoded on the clone C specific genomic island PACGI-1/TLPQC, in addition to their core genome homologs, contribute to protein quality control in SG17M. The ATP-dependent metalloprotease *ftsH2*, encoded on the genomic island, interacts and backs up the fitness-related functionalities of the core genome copy *ftsH1*. Both copies are required for optimal growth and expression of a multitude of cellular functions such as motility, biofilm formation, antibiotic resistance, secondary metabolites secretion and tolerance to elevated temperature and oxidative stress. The N-terminal 151 amino acids of FtsH1 – in particular, the periplasmic domain and the glycine-rich linker connecting the N-terminal region with the AAA+ module – are determinative factors for optimal *in vivo* functionality. The clone C specific genomic island encodes the disaggregases $ClpG_{GI}$ that, together with its homolog ClpG encoded on the core genome, confers superior heat tolerance to *P. aeruginosa* SG17M.

Furthermore, The core heat locus with *dna-shsp20*$_{GI}$*-clpG*$_{GI}$ confers heat tolerance to a newly characterized gastrointestinal *E. coli* isolate termed Fec10 that belongs to the ST10 MLST group. The $ClpG_{GI}$ disaggregase produced in SG17M and Fec10 provides heat tolerance and controls protein quality through their high basal ATPase activity and the robust disaggregation activity, which does not require a co-chaperone system. Another hallmark of the two most common clonal groups of *P. aeruginosa*, clone C strains and PA14 clone isolates with PA14 as a representative, is instant double crossover recombination upon conjugation of a gene replacement suicide vector. This observation facilitates genome engineering in these strains by omitting the counterselection step but also indicates a potential evolutionary selected trait of highly successful clonal groups.

6 FUTURE PERSPECTIVES

The horizontally acquired *ftsH2* protease copy encoded on the genomic island provides a backup functionality to the core genome copy *ftsH1* in *P. aeruginosa* SG17M. One question that needs to be answered is whether FtsH2 manifests a unique specific advantage to clone C. This thesis shows a first hint for a unique role of FtsH2, for example, FtsH2 is exclusively produced in the late stationary phase of growth, unlike FtsH1, which is hardly produced. Such a finding might indicate that FtsH2 is involved in protein quality control during stress conditions imposed by entering the late stationary phase of growth. This hypothesis can be further validated by investigating the fitness of the *ftsH2* deletion mutant under various stress conditions in the late stationary phase. For non-clone C strains of *P. aeruginosa* that encode one *ftsH* copy, *ftsH2* can be added on an expression vector and the strains can be tested for stress tolerance at the late stationary phase to demonstrate the unique contribution of *ftsH2*. Furthermore, the N- and the C-terminus of FtsH1 and FtsH2 are most divergent. We have already shown that the N-terminal amino acid sequence provides selective functionality *in vivo*. Another hint indicating that FtsH2 has potentially a selective repertoire of substrates is that candidate protein substrates were selectively pulled-down with $FtsH2^{trap}$, but not pulled-down with $FtsH1^{trap}$ (although the pull-down was not saturated). In addition, the hypothetical joined forces of FtsH2 with FtsH1 via hetero-oligomer complex formation might widen FtsH substrate selectivity and thereby functionality in *P. aeruginosa* SG17M. Therefore, it would be worth investigating a potential hetero-complex function *in vitro* by testing the *in vitro* enzymatic properties of an FtsH1/FtsH2 hetero-oligomeric complex. This can be achieved by first co-expressing *ftsH1* and *ftsH2* on the same expression vector, co-purifying the hetero-complexes and testing their *in vitro* enzymatic properties and compare it to the homo-oligomeric complexes. Consequently, localization of FtsH1 and FtsH2 in the inner membrane as homo- and hetero-oligomers will provide valuable information on the topology and arrangement of the subunits. This can be done, for example, by TEM using gold-labeled specific antisera raised against FtsH1 and FtsH2.

Surprisingly, the first N-terminal 151 amino acids of FtsH1 are sufficient for full complementation of the growth retardation phenotype of an *ftsH1* mutant with FtsH1 or a hybrid FtsH2 protein. Therefore, it is worth testing if the N-terminal domain also contributes to optimal activity *in vitro*, by checking the degradation activity of the chimeric constructs using relevant (to be identified) native substrates or the model substrates used in this thesis. The glycine-rich linker of the N-terminal region of FtsH1 was shown to be particularly determinative for *in vivo* functionality. To test if the linker alone without other N-terminal sub-domains of FtsH protease is functional to enhance the proteolytic activity of FtsH proteases, constructs with swapped linkers can be constructed and tested *in vitro* and *in vivo*.

ClpG disaggregases confer superior heat tolerance to *P. aeruginosa* and *E. coli* by binding aggregates via their N-terminal extension. The candidate substrates pulled-down with the $ClpG_{GI}$ variant with a double Walker B mutation (unpublished data, this work) indicate preliminarily that $ClpG_{GI}$ disaggregases might act on specific substrates that may not be necessarily aggregates (although this work has not directly provided experimental evidence for this scenario). Therefore, it would be interesting to confirm the candidate substrates *in vivo* and *in vitro* to extend $ClpG_{GI}$ functionality beyond the provision of thermotolerance. In conclusion, this thesis demonstrates the utter importance of the FtsH protease and ClpG disaggregase for the general fitness of *P. aeruginosa* and *E. coli*, making them potential druggable targets to develop novel antimicrobial and antivirulence agents (237).

7 ACKNOWLEDGMENTS

During my PhD journey away from home, I got to meet so many wonderful people that inspired me and helped me along this journey. I would like to say big **THANK YOU** to everyone I've encountered along the way.

First, I would like to express my gratitude to **Ute Römling**, my PhD principal supervisor, for allowing me to do my PhD in your lab. Thank you for teaching me resilience in science and how to evaluate my experimental data critically, for sharing your enthusiasm for science and newly published research. You got the best out of me towards a successful PhD with several publications and few in the pipeline; I wouldn't have done it if it wasn't for you. Your dedication to science and making sure of the success of your students are just what any graduate student would wish for. I have always admired your super quick response on manuscripts, forms, or questions via email, even during your busiest times. Often your work pace is way faster than anyone else in the lab. You are simply available for everyone, almost 24/7. I always see you on the bus working on manuscripts, replying to emails, or reading a scientific article. I remember once when we had a power outage at MTC, and you simply put a headlamp on and continued reading a paper (no excuses). Just small things that proved that you are a living example of ''science never sleeps''. Thank you for organizing Sunday writing workshops, where you, Fengyang and I met every Sunday morning for a few hours to practice writing manuscripts and discuss different writing styles. I have definitely learned a lot from these sessions; it made my thesis writing easier! I am grateful for your dedication. Thank you for encouraging me to try new things and travel to different labs (for almost one year) to collaborate with other researchers and to get a different perspective on my projects. For letting me test the waters outside academia through an internship at VR. For helping me remain focused and motivated during stressful times. You are always keen on doing pleasant outdoor activities with the group. Thank you for your interest in the culture and background of the people around you. Frankly, I can never thank you enough!!

I don't know where to start talking about my co-supervisor, **Changhan Lee**. Thank you for basically ''adopting me'' since my first day in the lab and almost four years after that of being a friend, a colleague and a mentor. Thank you for inspiring me in so many ways about science and life in general. Thank you for being there any time I needed you even after you left to Michigan. I am very grateful for your supervision. I have learned a lot from you on the professional and personal level. Thank you for sharing the candy care packages that Hyun-hee sent you from Korea ☺ (thank you, Hyun-hee for being a fantastic and nice colleague).

I would like to thank my current colleagues in the lab—my journey companion **Fengyang or Yo**, for being the most relaxed person. Your worry-free and funny attitude made it very enjoyable to work with you. We have been through a lot together, during which you have been nothing but a supportive colleague and a fantastic friend. **Jonatan or Johansson**, for sharing your knowledge about the dark-side cloning and for your ability to talk about the most random things ever. It was always lovely to have you around during my late night

working shifts; those were enlightening times ;). **Sulman**, for being supportive and for the friendly conversations over coffee. Thank you for being generous with your answers (sometime too generous :p). **Aswathy**, for being the newest addition to the *Pseudomonas* team.

I would like to thank my former lab mates—**Annika**, for your kind heart and taking good care of the lab and also your colleagues. Thanks for the life hack how to nail the SDS-PAGE preparation, works every time ☺. **Filip**, for being a curious person and always asking questions. **Lydia**, for your compassion. **Irfan**, for your scientific input. **Yousef**, for being friendly. **Inari**, for composing and playing songs in between incubation times. **Steffi**, for your contribution to my project. **Damien**, always fun to ''ketchup'' with you. **Lei,** for your dedication. **Karin**, for the pleasant conversation about the royal family and the dark humor of the British comedy. Also thanks to our lab neighbors at the old MTC building, **Patricia**, **Margareta** and **DaeHo**. Thank you **Roland Möllby** for your input on my grant application in Swedish.

Thank you, **Åsa Sjöling**, for being my PhD mentor, my book examiner and for always having time for me. It was nice hearing your perspective on things in and outside academia. Thank you, **Lisa Westerberg,** for being my yearly follow-up chairperson and providing advice on my academic progress. Thank you also for accepting to be my chairperson for my PhD defense. You are the only person I know that misses Boston's green line metro (aka the washing machine ☺).

Special thanks to my super smart collaborators who welcomed me in their labs and helped me expanding my projects. **Axel Mogk**, for hosting me in your lab at Heidelberg university twice and introducing me to the exciting world of biochemistry. I enjoyed working with you in the lab, and I hope I learned one or two things from your exceptional organizational skills and performing experiments meticulously (although starting experiments at 7:45 am was brutal ☺). Also, thank you for sharing your passion for football and inviting me over your place several times to watch the world cup. Also, thank you for lending me a bike to move around and for organizing the fantastic hiking trip. I would like to thank the other **Bukau** group members, **Pana, Areb, Lena, Svenja, Aseem, Declan and Regina,** for sharing your pipettes and bench spaces with me and making Heidelberg enjoyable. **Nina**, because of you, I only watch animal documentaries narrated by Snoop Dogg!! Thank you very much for the excellent time in Heidelberg and Boston and for merely being there whenever I need you! A big thanks for proofreading my thesis, Grazie!

I would like to sincerely thank the **Tania Baker research group** for hosting me in their lab at MIT for seven months. **Tania**, you are indeed an outstanding scientist and an exceptional human being. Thank you for generously sharing your life experience and exciting stories with me in the car on our way to work every morning. For being super thoughtful, supportive and warm-hearted towards the people around you. You treated me as a member of your lab, not as a guest researcher and I appreciate that. Thank you also for hosting me in the little house and for making me always feel at home. I appreciate it a lot! **Gina Mawla or Genius**, thank you

for your valuable help setting up the project, fixing protocols and always being a nice ‘’bay mate’’. **SaRa**, for your help with all the paperwork to make my trip to the US possible. Also, for our weekend strolls exploring Allstone and Brookline. **Sora**, for your generosity with materials and protocols and throwing the best Tiki party. **Hema**, for the insightful remarks on my project, the Chai breaks in the kitchen and the crash course on black Friday in the US. **Meghann**, for fun conversations about TV shows and chips flavors. **Kristin**, for tech talks and for sharing your protocols and always being ready to help out regardless of how busy you are. **Igor**, for sharing your passion for wilderness and European Christmas markets. **Irene**, for being sweet and kind. **Christina**, I don’t know how I was living without ballpoint pens before ;), it was always pleasant to chat with you about your love for MBTA! Thanks for your recommendations on what to do in Salem. Thank you guys for making Boston feel like my second home ☺.

My MTC friends and colleagues, you made my time as a PhD student easier and much more enjoyable. I am grateful for the exchange of stories, knowledge, experience, frustrations and jokes. Thank you, **Shawon** and **Afrouz,** for letting me use your centrifuge almost for free :P. **Afrouz**, you have been my study buddy, I appreciated that we prepared for the halftime review together. It was good to have you as a writing companion at stadsbiblioteket and KIB with lots of talks, writing and snacks (more like kebab-breaks ☺). **Leona**, for our career and life talks over many coffee breaks, thank you for being a good listener. **Silke**, for your help and tips with all the disputation paperwork, and the lovely Charles Riverwalk in Boston.

Johanna, for your persistence and grit finding your passion. **Ainhoa**, for being trustworthy and sincere. **Néstor**, for your energy and being true to yourself. **Marco**, for your sports goals and delightful conversation about swimming. **Lifeng**, for your tips on how to prepare for the dissertation and the lovely chat in the corridor. **Benedek**, for being adventurous and reminding me to do my tax return in Sweden and the US ☺. One day we maybe hit this mountain bike trail. **Marc**, for the pleasant conversations about life and career and the time at Pero theater. **Leo**, for your help with the shared equipment. **Ana Marin**, for the best thesis cover ever. **Bastian**, for showing interest in FtsH protease. **Ben**, for the lovely time at the gym. **Vanessa**, for your courageous soul to wandering around the globe by yourself. **Lidia**, for your enthusiasm and passion for science.

Anton, for sharing your experience about working as an MD in Sweden, **Wesam**, for our small talks in the corridor, good luck for the last bit of your PhD journey. **Lotta**, for nice lunch breaks. **Julian**, for keeping calm when I mistakenly congratulated you for your kid that you didn’t have! **Chris**, for your passion for Quentin Tarantino. **Azadeh**, for your genuine interest in people and showing compassion. **Manasa**, for a lovely chat each time we meet in the corridor. **Enrique**, for being so much fun to talk to, thanks for being supportive and kind. **Patrik**, for the lovely time in Boston and connecting me to your contacts when I needed help. **Jaime**, for the fantastic piano tunes on the weekends at Biomedicum. **Gerry**, for the fun random discussions about science and thesis covers. **Benedict**, it is always lovely to talk to

you about anything, thanks for keeping us entertained with stories when we shared the same corridor back at the old MTC building.

My colleagues on the other side (Bioclinicum), **Kim, Jens, Padryk, Vickey, Laura, and Jill**, thank you for scientific discussions and fun conversation at MTC events. Thanks to the **MSA** members for all the fun and social events. Other MTC people, **María Mushtaq, Marcus. Cajsa, Uta, Pontus, Nuno, Juliana, Ruining, Graciela, Carina, Mariana, Juan, Jonathan Coquet, Tony, Saadya, Monica, Madle and Tawana** thank you all for being friendly, helpful and simply wonderful colleagues.

I would like to thank the **Galina Selivanova group and Lars Gunnar group** for their generous help whenever we fall short on something in the lab and for the friendly chat in the corridor and over lunch. **J and Taka** from the **Yihai group**, thank you for the friendly chat by the coffee machine.

Thank you, **Gesan,** for your dedication to PhD students well-being and for always checking on everyone if everything is going alright. **Åsa**, thank you for showing a genuine interest in the people you meet, thanks for showing me how to find things on the KI website maze and being a fantastic student administrator, I've always enjoyed our conversation in Swedish. **Mia**, thanks for your prompt help with paperwork and insurance stuff. **Eva**, thank you so much for helping me with paperwork for my PhD application. **Magnus and Per**, for excellent after-work gym sessions and for being MTC superheroes.

I would also like to thank my friends from different departments at KI. **Eva**, for being very knowledgeable and humble, thanks for the lovely lunch breaks. **Joanne Bakker**, for your sense of humor and your taste in podcasts. **Mauricio**, for our basketball games. **Sara Padrell Sánchez**, for your enthusiasm about health and TomTom watches. **Carles**, for random career talks at the gym. **Karen**, for sharing your passion for ice skating and microbiology and for letting me use your lab equipment ☺. **Evan**, for your generosity in helping me out many times, I appreciate it a lot! **Tatiana**, for your fun personality and your activities outside the lab. **Cagla**, for the nice chat about science, apartment hunts and for being the best Danish ambassador. **Ewoud**, for your super awesome publication record and being very friendly every time we meet briefly. **Sandra Petrus**, for your passion for everything you do. **Ayla**, for your energy, wit, and super fun conversations, thank you for your efforts at the career service at KI, you are doing a great job.

I would like to thank the incredible team of KI career blog. Special thanks to **Natalie** for your enthusiasm for science communication, excellent writing tips, for introducing me to SSMF and helping me with my first and hopefully not last blog post ☺. Career service superheroes, **Anethe, Victoria, Ayla, and Kerstin** for your effort in organizing useful career events.

I am grateful for everyone I met during my internship at **Vetenskapsrådet (VR)** for helping me adapt to a new working culture away from the lab. Special thanks to the JPIAMR people,

Patriq, Shawon, Laura, Anders, and **Elisabeth,** for welcoming me to the VR family and helping me to get the most out of my internship.

Thank you, **Sharesta,** for being such a fantastic close friend and colleague! You are the first one I met when I came to Stockholm in 2014, we have been through a lot, and I appreciate your help and support all these years. Thanks for being very dependable whenever I needed you, and thanks for those Dutch chocolate sprinkles ☺. **Valentina**, for showing me new music and for the countless concerts we attended. Thank you for being a real, unique, good listener and one of my best friends. **Andreas**, for being super friendly and humble. **Nastya**, for your incredible musical talent and the postcards, spasibo!

My Forum family, **Fredrik, aka Freddy boy, Sofia, Malina, Uli, Anna, Simon and Anita**. Thank you for your care and support. We have lived together for several years, and it was simply amazing. Thanks for lots of outdoor events (downhill sledging in IKEA bags), board games (game of associations), movie nights (Cheesy Christmas movies that you force me to watch or long movies picked by Freddy that I sleep in the middle of it), concerts (I am from Barcelona and OH Wonder that you never told me about) and fantastic food (Uli takes time laps videos of things baking in the oven). You guys made it easier for me to live away from home. You are like my family in Stockholm. **Freddy**, I am very grateful to you, my friend. We have been through lots of ups and downs together and you have always been by my side. Thank you for always being willing to help out with anything. Thanks for being such a good friend. **Alex**, for being the best COVID-19 quarantine buddy with all we can eat pancake sessions. I appreciate your support during this hard time (of mainly being stuck at home with you :p). **Åsa**, for taking care of the kitchen plants and for the fun time in Boston.

To my Jägargatan friends, **Essi, Jone, Ninke, Naz, Valentine, Wiebke, Shawon, MJ, Majid, Manon, Gabs, Caitlin, Denis, Øyvind**. Thank you for the fantastic time together.

Till mina underbara surfvänner **Felicia, Sofia, Sandi, Marina, Victor, Greta, Marina, Oscar, Evelina och Hanna**. Stort tack för skratt, träning, resor, firande under midsommar och födelsedagar. Ni är fantastiska vänner och jag uppskattar verkligen er väldigt mycket.

Shout out to my favorite swimmers **Eva and James.** Thank you guys for our amazing AW swimming session, followed by a high carb meal ☺. I cherish our good memories together in Debaser, Trädgården and watching countless summer sunsets from Skinnarviksberget. **Bettina**, for being the best Norwegian host and saving my visa to Singapore.

Mahmoud, Bakr, and **Kareem,** my favorite trio-Egyptian friends, you guys are a big part of my life in Stockholm. Thanks for the fantastic memories over the years. **Sally**, you have no idea how much I am grateful for you, every time I meet you, even for brief minutes in the bus, you give me a new perspective or open up a unique opportunity for me. **Ahmed Elewa**, thanks for sharing your books with me and for the lovely time in Boston. **Shady**, for being genuinely nice, I enjoy talking to you about many things, it is crazy how similar we are in many ways. **Yousry**, you are one of the people that I like asking for opinions and advice.

Thank you for sharing your perspective in life with me, my friend. **Tonia**, for being very lovely and friendly. **Amir Shalaby**, for iftar at Amida and your place. My other Egyptian/Arabic friends in Stockholm, **Hesham, Mai, Maha, Thaer, Lamis, Sumo, Ramy Helal, Ramy Mansour, Rania,** and **Mahinor.** Thank you guys for making Stockholm feel somewhat like home with all the authentic food and pleasant company over the years.

Noha, thank you for the laughs in the lab, the crash course on what happened in Egypt since I left, and the unlimited supply of PBS for my experiments ☺. **Mohamed and Haneen,** thank you guys for your encouragement and support in the past few months. **Tamer**, for binge listening to Science Vs., talking about podcasts and training and for our lunch meditation sessions ;). **Loay and Abdel-rahman**, for being very lovely colleagues.

To my friends back in Egypt, **Mahdi, Raghda, Inji, Shafik, Assem, Safa, Marwa, Yara, Malak** and **Hector**. I miss you guys very much. **Mahdi**, for being my best friend who always got my back. Thank you for being you and everything you did for me. Despite the distance, you are the person that understands me the most. Thanks for your support and encouragement through this time, and basically, since I met you. **Raghda**, my trustworthy and reliable close friend. Thank you for your kindness and amazing heart, for giving me positive energy when I needed it the most and for visiting me in Stockholm. **Amr Hemeda**, thanks for your understanding and constant care. **Omar Hammam**, for your pure soul and excellent articulation skills.

Thanks to my home university, FUE, for supporting my studies abroad. Special thanks to **Mr. Khaled Azzazi** for this opportunity. Special thanks to my colleagues, **Dr. Marwa Raafat, Dr. Marwa Tammam, Dr. Amal, Dr. Yousra, Mona, Nada Anwar, Dina Khaled** and **Amr**. Thank you, **Dr. Seham, Dr. Manal, Dr. Ebada**, for your support. **Mrs. Gehan and Mahdi**, for your prompt and efficient help with the practical matters, any time I needed it.

To my AUC friends and professors, **Dr. Rania Syam, Dr. Ahmed Mostafa, Dr. Hamza El Dorry, Nahla, Laila, Sara, Hind, Mostafa and Mo'men**. Thank you for making me discover my passion for molecular biology. I appreciate all the scientific input.

Leonie, for your dedication and fighting spirit at the MSA and DSA to improve conditions for PhD students. Thank you for being diligent, dependable, and trustworthy. A big thank you for the awesome cover idea and helping me out bringing it to life (sorry for not having UFOs or aliens there, though ☺). Thank you for being the most kind, tender and thoughtful person. Thank you for proofreading my thesis and your valuable opinions along the way inside and outside science.

Jana und Lutz, die mich zweimal in Deutschland aufgenommen haben und die tollsten Gastgeber sind. Danke, dass ich meine Liebe "Maggi" entdecken durfte. Danke :).

To my family, you are my backbone and ultimate supporters. Special thanks to, **Khalo Ehab and Ramy**, for being role models to me since I was young. I still very much admire your ambition in life and daring attitude to take risks (all the time) to learn and grow. **Mostafa, Menna and tant Azza,** thanks for the support.

Mama, I know you are working very hard on your English language, so I know you will be happy and proud when you read and understand this (you can translate to baba :P). **Baba and mama**, Thank you very very very much for everything you have done for me all these years. Simply, I wouldn't become a doctor or even anything if it wasn't for your love and support. You still pick me up when I fall as you always did when I was a little kid. Thank you for supporting all my life decisions and trusting that I know what is best for myself. Thank you for not worrying about me too much. Thank you for listening, understanding and doing everything you can to help, although it might be hard sometimes due to the distance. Thank you for your lovely summer visits to Stockholm, I appreciate your yearly stock of ''Feter'' ☺. I miss you, and love you so much.

شكرا **بابا وماما** على كل ما فعلتموه من أجلي. لم أستطع الوصول إلى هذه المرحلة لولا دعمكم. كنتم تشجعونني دائمًا خلال هذه الرحلة على الرغم أنكم لم تكن تعرفون ما كنت أفعله بالضبط في المعمل. أنا آسف لأنني لم أحاول جاهداً ما يكفي لشرح مشروع بحثي. ولكن الآن منذ ان طبعت هذا الكتاب اخيرًا، يمكنني أن أشرح لكم بالتفصيل الممل أشكركم على تحمل غيابي كل هذه السنوات. كنت دائما سعيدا عندما كنتم تزورني في السويد.
شكرا لسماعكم و مشاركتكم افراحي وهمومي و محاولتكم لحل المشاكل بالرغم من طول المسافات.
افتقدكم وأحبكم كثيرا!!!

Thank you all!!

8 REFERENCES

1. Y. Carmeli, N. Troillet, G. M. Eliopoulos, M. H. Samore, Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. *Antimicrobial agents and chemotherapy* **43**, 1379-1382 (1999).
2. K. G. Kerr, A. M. Snelling, *Pseudomonas aeruginosa*: a formidable and ever-present adversary. *Journal of Hospital Infection* **73**, 338-344 (2009).
3. M. D. Parkins, R. Somayaji, V. J. Waters, Epidemiology, Biology, and Impact of Clonal *Pseudomonas aeruginosa* Infections in Cystic Fibrosis. *Clin Microbiol Rev* **31** (2018).
4. J. B. Lyczak, C. L. Cannon, G. B. Pier, Lung infections associated with cystic fibrosis. *Clinical microbiology reviews* **15**, 194-222 (2002).
5. J. W. Bendig, P. W. Kyle, P. L. Giangrande, D. M. Samson, B. S. Azadian, Two neutropenic patients with multiple resistant *Pseudomonas aeruginosa* septicaemia treated with ciprofloxacin. *J R Soc Med* **80**, 316-317 (1987).
6. M. Kielhofner, R. L. Atmar, R. J. Hamill, D. M. Musher, Life-Threatening *Pseudomonas aeruginosa* Infections in Patients with Human Immunodeficiency Virus Infection. *Clinical Infectious Diseases* **14**, 403-411 (1992).
7. J. B. Lyczak, C. L. Cannon, G. B. Pier, Establishment of *Pseudomonas aeruginosa* infection: lessons from a versatile opportunist. *Microbes Infect* **2**, 1051-1060 (2000).
8. J. F. Silvestre, M. I. Betlloch, Cutaneous manifestations due to *Pseudomonas* infection. *Int J Dermatol* **38**, 419-431 (1999).
9. J. B. Goldberg, '*Pseudomonas* '99, The Seventh International Congress on Pseudomonas: Biotechnology and Pathogenesis', organized by the American Society for Microbiology, was held in Maui, HI, USA, 1–5 September 1999. *Trends in Microbiology* **8**, 55-57 (2000).
10. L. Wiehlmann, N. Cramer, B. Tümmler, Habitat-associated skew of clone abundance in the *Pseudomonas aeruginosa* population. *Environmental Microbiology Reports* **7**, 955-960 (2015).
11. P. S. Stewart, J. William Costerton, Antibiotic resistance of bacteria in biofilms. *The Lancet* **358**, 135-138 (2001).
12. N. Hoiby, O. Ciofu, T. Bjarnsholt, *Pseudomonas aeruginosa* biofilms in cystic fibrosis. *Future Microbiol* **5**, 1663-1674 (2010).
13. J. W. Costerton, P. S. Stewart, E. P. Greenberg, Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322 (1999).
14. L. Hall-Stoodley, J. W. Costerton, P. Stoodley, Bacterial biofilms: from the natural environment to infectious diseases. *Nature reviews. Microbiology* **2**, 95-108 (2004).
15. R. Simm, I. Ahmad, M. Rhen, S. Le Guyon, U. Romling, Regulation of biofilm formation in *Salmonella enterica* serovar Typhimurium. *Future Microbiol* **9**, 1261-1282 (2014).
16. R. J. McLean, M. Whiteley, D. J. Stickler, W. C. Fuqua, Evidence of autoinducer activity in naturally occurring biofilms. *FEMS Microbiol Lett* **154**, 259-263 (1997).
17. S. Mukherjee *et al.*, The PqsE and RhlR proteins are an autoinducer synthase-receptor pair that control virulence and biofilm development in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **115**, E9411-e9418 (2018).
18. G. A. O'Toole, G. C. Wong, Sensational biofilms: surface sensing in bacteria. *Current opinion in microbiology* **30**, 139-146 (2016).
19. K. B. Barken *et al.*, Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms. *Environmental microbiology* **10**, 2331-2343 (2008).
20. R. E. Hancock, Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin Infect Dis* **27 Suppl 1**, S93-99 (1998).
21. B. D. Hoyle, J. W. Costerton, Bacterial resistance to antibiotics: the role of biofilms. *Prog Drug Res* **37**, 91-105 (1991).
22. E. B. Breidenstein, C. de la Fuente-Nunez, R. E. Hancock, *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* **19**, 419-426 (2011).
23. L. Fernandez, R. E. Hancock, Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* **25**, 661-681 (2012).
24. T. I. Nicas, R. E. Hancock, *Pseudomonas aeruginosa* outer membrane permeability: isolation of a porin protein F-deficient mutant. *J Bacteriol* **153**, 281-285 (1983).
25. X. Z. Li, H. Nikaido, K. Poole, Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **39**, 1948-1953 (1995).
26. S. Sandoval-Motta, M. Aldana, Adaptive resistance to antibiotics in bacteria: a systems biology perspective. *Wiley Interdiscip Rev Syst Biol Med* **8**, 253-267 (2016).

27. R. L. Gibson, J. L. Burns, B. W. Ramsey, Pathophysiology and management of pulmonary infections in cystic fibrosis. *American journal of respiratory and critical care medicine* **168**, 918-951 (2003).
28. T. R. Costa *et al.*, Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol* **13**, 343-359 (2015).
29. A. R. Hauser, The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* **7**, 654-665 (2009).
30. S. Bleves *et al.*, Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons. *International journal of medical microbiology : IJMM* **300**, 534-543 (2010).
31. A. A. Angus, D. J. Evans, J. T. Barbieri, S. M. Fleiszig, The ADP-ribosylation domain of *Pseudomonas aeruginosa* ExoS is required for membrane bleb niche formation and bacterial survival within epithelial cells. *Infection and immunity* **78**, 4500-4510 (2010).
32. V. Finck-Barbancon *et al.*, ExoU expression by *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. *Molecular microbiology* **25**, 547-557 (1997).
33. S. M. Fleiszig *et al.*, *Pseudomonas aeruginosa*-mediated cytotoxicity and invasion correlate with distinct genotypes at the loci encoding exoenzyme S. *Infection and immunity* **65**, 579-586 (1997).
34. L. Chen, Y. Zou, P. She, Y. Wu, Composition, function, and regulation of T6SS in *Pseudomonas aeruginosa. Microbiological research* **172**, 19-25 (2015).
35. G. W. Lau, D. J. Hassett, H. Ran, F. Kong, The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends in molecular medicine* **10**, 599-606 (2004).
36. L. E. Dietrich, A. Price-Whelan, A. Petersen, M. Whiteley, D. K. Newman, The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* **61**, 1308-1321 (2006).
37. P. N. Jimenez *et al.*, The multiple signaling systems regulating virulence in *Pseudomonas aeruginosa*. *Microbiol Mol Biol Rev* **76**, 46-65 (2012).
38. S. Jayaseelan, D. Ramaswamy, S. Dharmaraj, Pyocyanin: production, applications, challenges and new insights. *World J Microbiol Biotechnol* **30**, 1159-1168 (2014).
39. Q. Cui *et al.*, Cross-Regulation between the *phz1* and *phz2* Operons Maintain a Balanced Level of Phenazine Biosynthesis in *Pseudomonas aeruginosa* PAO1. *PloS one* **11**, e0144447 (2016).
40. D. A. Recinos *et al.*, Redundant phenazine operons in *Pseudomonas aeruginosa* exhibit environment-dependent expression and differential roles in pathogenicity. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 19420-19425 (2012).
41. P. Visca, F. Imperi, I. L. Lamont, Pyoverdine siderophores: from biogenesis to biosignificance. *Trends Microbiol* **15**, 22-30 (2007).
42. P. Cornelis, J. Dingemans, *Pseudomonas aeruginosa* adapts its iron uptake strategies in function of the type of infections. *Front Cell Infect Microbiol* **3**, 75 (2013).
43. I. L. Lamont, P. A. Beare, U. Ochsner, A. I. Vasil, M. L. Vasil, Siderophore-mediated signaling regulates virulence factor production in *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 7072-7077 (2002).
44. J. Kramer, O. Ozkaya, R. Kummerli, Bacterial siderophores in community and host interactions. *Nat Rev Microbiol* **18**, 152-163 (2020).
45. H. Budzikiewicz, Siderophores of the *Pseudomonadaceae sensu stricto* (fluorescent and non-fluorescent *Pseudomonas* spp.). *Fortschritte der Chemie organischer Naturstoffe = Progress in the chemistry of organic natural products. Progres dans la chimie des substances organiques naturelles* **87**, 81-237 (2004).
46. N. Folschweiller *et al.*, The interaction between pyoverdin and its outer membrane receptor in *Pseudomonas aeruginosa* leads to different conformers: a time-resolved fluorescence study. *Biochemistry* **41**, 14591-14601 (2002).
47. D. Grothues, B. Tümmler, Genome analysis of *Pseudomonas aeruginosa* by field inversion gel electrophoresis. *FEMS Microbiology Letters* **48**, 419-422 (1987).
48. U. Römling *et al.*, Epidemiology of chronic *pseudomonas aeruginosa* infections in cystic fibrosis. *Journal of Infectious Diseases* **170**, 1616-1621 (1994).
49. L. Wiehlmann *et al.*, Population structure of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **104**, 8101-8106 (2007).
50. M. C. Maiden *et al.*, Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc Natl Acad Sci U S A* **95**, 3140-3145 (1998).
51. J. P. Pirnay *et al.*, *Pseudomonas aeruginosa* population structure revisited. *PLoS One* **4**, e7740 (2009).
52. H. Tettelin *et al.*, Genome analysis of multiple pathogenic isolates of *Streptococcus* agalactiae: implications for the microbial "pan-genome". *Proc Natl Acad Sci U S A* **102**, 13950-13955 (2005).
53. B. Valot *et al.*, What It Takes to Be a *Pseudomonas aeruginosa*? The Core Genome of the Opportunistic Pathogen Updated. *PLoS One* **10**, e0126468 (2015).

54. J. Mosquera-Rendon *et al.*, Pangenome-wide and molecular evolution analyses of the *Pseudomonas aeruginosa* species. *BMC Genomics* **17**, 45 (2016).
55. L. Freschi *et al.*, The P*seudomonas aeruginosa* Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. *Genome Biol Evol* **11**, 109-120 (2019).
56. E. A. Ozer, E. Nnah, X. Didelot, R. J. Whitaker, A. R. Hauser, The Population Structure of *Pseudomonas aeruginosa* Is Characterized by Genetic Isolation of *exoU*+ and *exoS*+ Lineages. *Genome Biol Evol* **11**, 1780-1796 (2019).
57. W. Arber, Genetic variation: molecular mechanisms and impact on microbial evolution. *FEMS microbiology reviews* **24**, 1-7 (2000).
58. V. L. Kung, E. A. Ozer, A. R. Hauser, The accessory genome of P*seudomonas aeruginosa*. *Microbiology and molecular biology reviews : MMBR* **74**, 621-641 (2010).
59. J. Hacker, J. B. Kaper, Pathogenicity islands and the evolution of microbes. *Annual review of microbiology* **54**, 641-679 (2000).
60. J. He *et al.*, The broad host range pathogen *Pseudomonas aeruginosa* strain PA14 carries two pathogenicity islands harboring plant and animal virulence genes. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 2530-2535 (2004).
61. S. Fischer *et al.*, Intraclonal genome diversity of the major *Pseudomonas aeruginosa* clones C and PA14. *Environmental microbiology reports* **8**, 227-234 (2016).
62. B. W. Holloway, Genetic recombination in *Pseudomonas aeruginosa*. *J Gen Microbiol* **13**, 572-581 (1955).
63. L. G. Rahme *et al.*, Common virulence factors for bacterial pathogenicity in plants and animals. *Science* **268**, 1899-1902 (1995).
64. B. G. Spratt, Exploring the concept of clonality in bacteria. *Methods in molecular biology (Clifton, N.J.)* **266**, 323-352 (2004).
65. B. J. Shapiro, How clonal are bacteria over time? *Curr Opin Microbiol* **31**, 116-123 (2016).
66. A. De Soyza *et al.*, Developing an international *Pseudomonas aeruginosa* reference panel. *MicrobiologyOpen* **2**, 1010-1023 (2013).
67. U. Römling, J. Wingender, H. Müller, B. Tümmler, A major *Pseudomonas aeruginosa* clone common to patients and aquatic habitats. *Appl Environ Microbiol* **60**, 1734-1738 (1994).
68. U. Romling, A. Kader, D. D. Sriramulu, R. Simm, G. Kronvall, Worldwide distribution of *Pseudomonas aeruginosa* clone C strains in the aquatic environment and cystic fibrosis patients. *Environmental microbiology* **7**, 1029-1038 (2005).
69. E. Rakhimova *et al.*, P*seudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. *The Journal of infectious diseases* **200**, 1928-1935 (2009).
70. P. Tielen *et al.*, Genotypic and phenotypic characterization of *Pseudomonas aeruginosa* isolates from urinary tract infections. *International journal of medical microbiology : IJMM* **301**, 282-292 (2011).
71. J. P. Pirnay *et al.*, Global *Pseudomonas aeruginosa* biodiversity as reflected in a Belgian river. *Environ Microbiol* **7**, 969-980 (2005).
72. K. Selezska *et al.*, *Pseudomonas aeruginosa* population structure revisited under environmental focus: impact of water quality and phage pressure. *Environ Microbiol* **14**, 1952-1967 (2012).
73. S. M. Petit *et al.*, Lagooning of wastewaters favors dissemination of clinically relevant *Pseudomonas aeruginosa*. *Research in microbiology* **164**, 856-866 (2013).
74. C. Lee *et al.*, A novel protein quality control mechanism contributes to heat shock resistance of worldwide-distributed *Pseudomonas aeruginosa* clone C strains. *Environ Microbiol* **17**, 4511-4526 (2015).
75. U. Romling, K. D. Schmidt, B. Tummler, Large genome rearrangements discovered by the detailed analysis of 21 *Pseudomonas aeruginosa* clone C isolates found in environment and disease habitats. *Journal of molecular biology* **271**, 386-404 (1997).
76. J. Klockgether, O. Reva, K. Larbig, B. Tummler, Sequence analysis of the mobile genome island pKLC102 of *Pseudomonas aeruginosa* C. *Journal of bacteriology* **186**, 518-534 (2004).
77. J. Klockgether, D. Wurdemann, O. Reva, L. Wiehlmann, B. Tummler, Diversity of the abundant pKLC102/PAGI-2 family of genomic islands in P*seudomonas aeruginosa*. *Journal of bacteriology* **189**, 2443-2459 (2007).
78. K. D. Larbig *et al.*, Gene islands integrated into tRNA(Gly) genes confer genome diversity on a *Pseudomonas aeruginosa* clone. *Journal of bacteriology* **184**, 6665-6680 (2002).
79. U. Römling, A. Kader, D. D. Sriramulu, R. Simm, G. Kronvall, Worldwide distribution of *Pseudomonas aeruginosa* clone C strains in the aquatic environment and cystic fibrosis patients. *Environ Microbiol* **7**, 1029-1038 (2005).
80. C. Lee, V. Peters, O. Melefors, U. Romling, Draft Genome Sequence of *Pseudomonas aeruginosa* SG17M, an Environmental Isolate Belonging to Clone C, Prevalent in Patients and Aquatic Habitats. *Genome announcements* **2** (2014).

81. M. S. Bojer, C. Struve, H. Ingmer, D. S. Hansen, K. A. Krogfelt, Heat resistance mediated by a new plasmid encoded Clp ATPase, ClpK, as a possible novel mechanism for nosocomial persistence of *Klebsiella pneumoniae*. *PloS one* **5**, e15467 (2010).
82. R. G. Mercer *et al.*, Genetic determinants of heat resistance in *Escherichia coli*. *Front Microbiol* **6**, 932 (2015).
83. C. Lee, E. Wigren, H. Lünsdorf, U. Römling, Protein homeostasis-more than resisting a hot bath. *Curr Opin Microbiol* **30**, 147-154 (2016).
84. S. M. Kamal *et al.*, Two FtsH Proteases Contribute to Fitness and Adaptation of *Pseudomonas aeruginosa* Clone C Strains. *Front Microbiol* **10**, 1372 (2019).
85. F. Orskov *et al.*, Special *Escherichia coli* serotypes among enterotoxigenic strains from diarrhoea in adults and children. *Med Microbiol Immunol* **162**, 73-80 (1976).
86. F. Orskov, I. Orskov, *Escherichia coli* serotyping and disease in man and animals. *Can J Microbiol* **38**, 699-704 (1992).
87. O. Tenaillon, D. Skurnik, B. Picard, E. Denamur, The population genetics of commensal *Escherichia coli*. *Nat Rev Microbiol* **8**, 207-217 (2010).
88. R. K. Selander *et al.*, Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873-884 (1986).
89. P. Goullet, B. Picard, Comparative electrophoretic polymorphism of esterases and other enzymes in *Escherichia coli*. *J Gen Microbiol* **135**, 135-143 (1989).
90. P. Desjardins, B. Picard, B. Kaltenbock, J. Elion, E. Denamur, Sex in *Escherichia coli* does not disrupt the clonal structure of the population: evidence from random amplified polymorphic DNA and restriction-fragment-length polymorphism. *J Mol Evol* **41**, 440-448 (1995).
91. E. M. Ribot *et al.*, Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog Dis* **3**, 59-67 (2006).
92. P. M. Fratamico *et al.*, Advances in Molecular Serotyping and Subtyping of *Escherichia coli*. *Front Microbiol* **7**, 644 (2016).
93. O. Clermont, S. Bonacorsi, E. Bingen, Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl Environ Microbiol* **66**, 4555-4558 (2000).
94. O. Clermont, J. K. Christenson, E. Denamur, D. M. Gordon, The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. *Environ Microbiol Rep* **5**, 58-65 (2013).
95. A. R. Manges *et al.*, Global Extraintestinal Pathogenic *Escherichia coli* (ExPEC) Lineages. *Clin Microbiol Rev* **32** (2019).
96. Z. D. Blount, The unexhausted potential of *E. coli*. *Elife* **4** (2015).
97. D. E. Chang *et al.*, Carbon nutrition of *Escherichia coli* in the mouse intestine. *Proc Natl Acad Sci U S A* **101**, 7427-7432 (2004).
98. C. Beloin, A. Roux, J. M. Ghigo, *Escherichia coli* biofilms. *Curr Top Microbiol Immunol* **322**, 249-289 (2008).
99. O. Koren *et al.*, Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell* **150**, 470-480 (2012).
100. H. J. Sears, I. Brownlee, J. K. Uchiyama, Persistence of individual strains of *Escherichia coli* in the intestinal tract of man. *J Bacteriol* **59**, 293-301 (1950).
101. M. A. Savageau, *Escherichia coli* Habitats, Cell Types, and Molecular Mechanisms of Gene Control. *The American Naturalist* **122**, 732-744 (1983).
102. R. R. Bollinger *et al.*, Human secretory immunoglobulin A may contribute to biofilm formation in the gut. *Immunology* **109**, 580-587 (2003).
103. R. Bentley, R. Meganathan, Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol Rev* **46**, 241-280 (1982).
104. J. G. Lawrence, J. R. Roth, Evolution of coenzyme B12 synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. *Genetics* **142**, 11-24 (1996).
105. T. A. Russo, J. R. Johnson, Medical and economic impact of extraintestinal infections due to *Escherichia coli*: focus on an increasingly important endemic problem. *Microbes Infect* **5**, 449-456 (2003).
106. J. B. Kaper, J. P. Nataro, H. L. Mobley, Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2**, 123-140 (2004).
107. B. J. Bachmann, Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriol Rev* **36**, 525-557 (1972).
108. P. Daegelen, F. W. Studier, R. E. Lenski, S. Cure, J. F. Kim, Tracing ancestors and relatives of *Escherichia coli* B, and the derivation of B strains REL606 and BL21(DE3). *J Mol Biol* **394**, 634-643 (2009).
109. T. Escherich, The intestinal bacteria of the neonate and breast-fed infant. 1884. *Rev Infect Dis* **10**, 1220-1225 (1988).

110. V. Khetrapal, K. S. Mehershahi, S. L. Chen, Complete Genome Sequence of the Original *Escherichia coli* Isolate, Strain NCTC86. *Genome Announc* **5** (2017).
111. H. Li, M. Ganzle, Some Like It Hot: Heat Resistance of *Escherichia coli* in Food. *Front Microbiol* **7**, 1763 (2016).
112. J. Gajdosova *et al.*, Analysis of the DNA region mediating increased thermotolerance at 58 degrees C in *Cronobacter sp.* and other enterobacterial strains. *Antonie Van Leeuwenhoek* **100**, 279-289 (2011).
113. E. A. Dlusskaya, L. M. McMullen, M. G. Ganzle, Characterization of an extremely heat-resistant *Escherichia coli* obtained from a beef processing facility. *J Appl Microbiol* **110**, 840-849 (2011).
114. S. V. Nguyen, G. P. Harhay, J. L. Bono, T. P. Smith, D. M. Harhay, Genome Sequence of the Thermotolerant Foodborne Pathogen *Salmonella enterica* Serovar Senftenberg ATCC 43845 and Phylogenetic Analysis of Loci Encoding Increased Protein Quality Control Mechanisms. *mSystems* **2** (2017).
115. E. J. Boll *et al.*, Turn Up the Heat-Food and Clinical *Escherichia coli* Isolates Feature Two Transferrable Loci of Heat Resistance. *Front Microbiol* **8**, 579 (2017).
116. E. J. Boll, J. Frimodt-Moller, B. Olesen, K. A. Krogfelt, C. Struve, Heat resistance in extended-spectrum beta-lactamase-producing *Escherichia coli* may favor environmental survival in a hospital setting. *Res Microbiol* **167**, 345-349 (2016).
117. A. Mogk, D. Huber, B. Bukau, Integrating protein homeostasis strategies in prokaryotes. *Cold Spring Harb Perspect Biol* **3** (2011).
118. K. L. Ewalt, J. P. Hendrick, W. A. Houry, F. U. Hartl, *In vivo* observation of polypeptide flux through the bacterial chaperonin system. *Cell* **90**, 491-500 (1997).
119. S. A. Teter *et al.*, Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains. *Cell* **97**, 755-765 (1999).
120. A. Gragerov *et al.*, Cooperation of GroEL/GroES and DnaK/DnaJ heat shock proteins in preventing protein misfolding in *Escherichia coli*. *Proc Natl Acad Sci U S A* **89**, 10341-10344 (1992).
121. P. Goloubinoff, A. Mogk, A. P. Zvi, T. Tomoyasu, B. Bukau, Sequential mechanism of solubilization and refolding of stable protein aggregates by a bichaperone network. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 13732-13737 (1999).
122. K. Motohashi, Y. Watanabe, M. Yohda, M. Yoshida, Heat-inactivated proteins are rescued by the DnaK.J-GrpE set and ClpB chaperones. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 7184-7189 (1999).
123. M. Zolkiewski, ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. A novel multi-chaperone system from *Escherichia coli*. *J Biol Chem* **274**, 28083-28086 (1999).
124. M. Haslbeck, T. Franzmann, D. Weinfurtner, J. Buchner, Some like it hot: the structure and function of small heat-shock proteins. *Nat Struct Mol Biol* **12**, 842-846 (2005).
125. A. Mogk, E. Deuerling, S. Vorderwulbecke, E. Vierling, B. Bukau, Small heat shock proteins, ClpB and the DnaK system form a functional triade in reversing protein aggregation. *Mol Microbiol* **50**, 585-595 (2003).
126. K. H. Swamy, A. L. Goldberg, *E. coli* contains eight soluble proteolytic activities, one being ATP dependent. *Nature* **292**, 652-654 (1981).
127. R. T. Sauer, T. A. Baker, AAA+ proteases: ATP-fueled machines of protein destruction. *Annu Rev Biochem* **80**, 587-612 (2011).
128. L. M. Iyer, D. D. Leipe, E. V. Koonin, L. Aravind, Evolutionary history and higher order classification of AAA+ ATPases. *J Struct Biol* **146**, 11-31 (2004).
129. A. F. Neuwald, L. Aravind, J. L. Spouge, E. V. Koonin, AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9**, 27-43 (1999).
130. T. Ogura, A. J. Wilkinson, AAA+ superfamily ATPases: common structure--diverse function. *Genes Cells* **6**, 575-597 (2001).
131. M. Saraste, P. R. Sibbald, A. Wittinghofer, The P-loop--a common motif in ATP- and GTP-binding proteins. *Trends Biochem Sci* **15**, 430-434 (1990).
132. J. E. Walker, M. Saraste, M. J. Runswick, N. J. Gay, Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo j* **1**, 945-951 (1982).
133. J. Snider, W. A. Houry, AAA+ proteins: diversity in function, similarity in structure. *Biochem Soc Trans* **36**, 72-77 (2008).
134. D. D. Leipe, E. V. Koonin, L. Aravind, Evolution and classification of P-loop kinases and related proteins. *J Mol Biol* **333**, 781-815 (2003).
135. M. P. Mayer, B. Bukau, Hsp70 chaperones: Cellular functions and molecular mechanism. *Cellular and Molecular Life Sciences* **62**, 670 (2005).

136. A. Mogk, B. Bukau, H. H. Kampinga, Cellular Handling of Protein Aggregates by Disaggregation Machines. *Mol Cell* **69**, 214-226 (2018).
137. F. D. Schramm, K. Schroeder, K. Jonas, Protein aggregation in bacteria. *FEMS Microbiol Rev* **44**, 54-72 (2020).
138. C. Lee *et al.*, Stand-alone ClpG disaggregase confers superior heat tolerance to bacteria. *Proc Natl Acad Sci U S A* **115**, E273-E282 (2018).
139. P. Katikaridis, L. Meins, S. M. Kamal, U. Romling, A. Mogk, ClpG Provides Increased Heat Resistance by Acting as Superior Disaggregase. *Biomolecules* **9** (2019).
140. E. Kruger, U. Volker, M. Hecker, Stress induction of *clpC* in Bacillus subtilis and its involvement in stress tolerance. *J Bacteriol* **176**, 3360-3367 (1994).
141. E. Kruger, E. Witt, S. Ohlmeier, R. Hanschke, M. Hecker, The *clp* proteases of Bacillus subtilis are directly involved in degradation of misfolded proteins. *J Bacteriol* **182**, 3259-3265 (2000).
142. T. Schlothauer, A. Mogk, D. A. Dougan, B. Bukau, K. Turgay, MecA, an adaptor protein necessary for ClpC chaperone activity. *Proc Natl Acad Sci U S A* **100**, 2306-2311 (2003).
143. J. D. Kowit, A. L. Goldberg, Intermediate steps in the degradation of a specific abnormal protein in *Escherichia coli*. *J Biol Chem* **252**, 8350-8357 (1977).
144. L. M. Bittner, J. Arends, F. Narberhaus, Mini review: ATP-dependent proteases in bacteria. *Biopolymers* **105**, 505-517 (2016).
145. M. D. Brazas, E. B. Breidenstein, J. Overhage, R. E. Hancock, Role of lon, an ATP-dependent protease homolog, in resistance of P*seudomonas aeruginosa* to ciprofloxacin. *Antimicrobial agents and chemotherapy* **51**, 4276-4283 (2007).
146. E. B. Breidenstein, B. K. Khaira, I. Wiegand, J. Overhage, R. E. Hancock, Complex ciprofloxacin resistome revealed by screening a *Pseudomonas aeruginosa* mutant library for altered susceptibility. *Antimicrobial agents and chemotherapy* **52**, 4486-4491 (2008).
147. A. K. Marr, J. Overhage, M. Bains, R. E. Hancock, The Lon protease of P*seudomonas aeruginosa* is induced by aminoglycosides and is involved in biofilm formation and motility. *Microbiology* **153**, 474-482 (2007).
148. L. Fernandez, E. B. Breidenstein, D. Song, R. E. Hancock, Role of intracellular proteases in the antibiotic resistance, motility, and biofilm formation of *Pseudomonas aeruginosa*. *Antimicrobial agents and chemotherapy* **56**, 1128-1132 (2012).
149. A. Takaya, F. Tabuchi, H. Tsuchiya, E. Isogai, T. Yamamoto, Negative regulation of quorum-sensing systems in *Pseudomonas aeruginosa* by ATP-dependent Lon protease. *Journal of bacteriology* **190**, 4181-4188 (2008).
150. L. M. Bittner, J. Arends, F. Narberhaus, When, how and why? Regulated proteolysis by the essential FtsH protease in *Escherichia coli*. *Biol Chem* **398**, 625-635 (2017).
151. C. Herman, D. Thevenet, P. Bouloc, G. C. Walker, R. D'Ari, Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev* **12**, 1348-1355 (1998).
152. J. M. Flynn *et al.*, Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 10584-10589 (2001).
153. E. Gur, R. T. Sauer, Recognition of misfolded proteins by Lon, a AAA(+) protease. *Genes & development* **22**, 2267-2277 (2008).
154. N. J. Kuhlmann, P. Chien, Selective adaptor dependent protein degradation in bacteria. *Curr Opin Microbiol* **36**, 118-127 (2017).
155. K. H. Wang, G. Roman-Hernandez, R. A. Grant, R. T. Sauer, T. A. Baker, The Molecular Basis of N-End Rule Recognition. *Molecular Cell* **32**, 406-414 (2008).
156. R. Schmidt, R. Zahn, B. Bukau, A. Mogk, ClpS is the recognition component for *Escherichia coli* substrates of the N-end rule degradation pathway. *Mol Microbiol* **72**, 506-517 (2009).
157. E. Gur, D. Biran, E. Z. Ron, Regulated proteolysis in Gram-negative bacteria--how and when? *Nat Rev Microbiol* **9**, 839-848 (2011).
158. W. Schumann, FtsH – a single-chain charonin? *FEMS Microbiology Reviews* **23**, 1-11 (1999).
159. K. Ito, Y. Akiyama (2005) Cellular functions, mechanism of action, and regulation of FtsH protease. in *Annual Review of Microbiology*, pp 211-231.
160. T. Ogura *et al.*, Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*. *Molecular microbiology* **31**, 833-844 (1999).
161. E. Deuerling, A. Mogk, C. Richter, M. Purucker, W. Schumann, The ftsH gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. *Mol Microbiol* **23**, 921-933 (1997).

162. T. Langer, AAA proteases: cellular machines for degrading membrane proteins. *Trends in Biochemical Sciences* **25**, 247-251 (2000).
163. G. Casari *et al.*, Spastic Paraplegia and OXPHOS Impairment Caused by Mutations in Paraplegin, a Nuclear-Encoded Mitochondrial Metalloprotease. *Cell* **93**, 973-983 (1998).
164. T. Tomoyasu *et al.*, Topology and subcellular localization of FtsH protein in *Escherichia coli*. *Journal of bacteriology* **175**, 1352-1357 (1993).
165. Y. Akiyama, T. Yoshihisa, K. Ito, FtsH, a membrane-bound ATPase, forms a complex in the cytoplasmic membrane of *Escherichia coli*. *J Biol Chem* **270**, 23485-23490 (1995).
166. Y. Akiyama, A. Kihara, H. Mori, T. Ogura, K. Ito, Roles of the periplasmic domain of *Escherichia coli* FtsH (HflB) in protein interactions and activity modulation. *J Biol Chem* **273**, 22326-22333 (1998).
167. S. Krzywda *et al.*, The Crystal Structure of the AAA Domain of the ATP-Dependent Protease FtsH of *Escherichia coli* at 1.5 Å Resolution. *Structure* **10**, 1073-1083 (2002).
168. H. Niwa, D. Tsuchiya, H. Makyio, M. Yoshida, K. Morikawa, Hexameric ring crystal structure of the ATPase domain of the membrane-integrated metalloprotease FtsH from Thermus thermophilus HB8. *Structure* **10**, 1415-1423 (2002).
169. C. Bieniossek *et al.*, The molecular architecture of the metalloprotease FtsH. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3066 (2006).
170. T. Tomoyasu *et al.*, The *Escherichia coli* FtsH protein is a prokaryotic member of a protein family of putative ATPases involved in membrane functions, cell cycle control, and gene expression. *J Bacteriol* **175**, 1344-1351 (1993).
171. F. Scharfenberg *et al.*, Structure and evolution of N-domains in AAA metalloproteases. *J Mol Biol* **427**, 910-923 (2015).
172. J. Snider, G. Thibault, W. A. Houry, The AAA+ superfamily of functionally diverse proteins. *Genome biology* **9**, 216 (2008).
173. K. Karata, T. Inagawa, A. J. Wilkinson, T. Tatsuta, T. Ogura, Dissecting the role of a conserved motif (the second region of homology) in the AAA family of ATPases. Site-directed mutagenesis of the ATP-dependent protease FtsH. *The Journal of biological chemistry* **274**, 26225-26232 (1999).
174. K. Karata, C. S. Verma, A. J. Wilkinson, T. Ogura, Probing the mechanism of ATP hydrolysis and substrate translocation in the AAA protease FtsH by modelling and mutagenesis. *Molecular microbiology* **39**, 890-903 (2001).
175. N. D. Rawlings, G. Salvesen, *Handbook of proteolytic enzymes* (Amsterdam : Elsevier, Amsterdam, ed. Third edition.. 2013).
176. Y. Akiyama, K. Ito, Roles of multimerization and membrane association in the proteolytic functions of FtsH (HflB). *Embo j* **19**, 3888-3895 (2000).
177. Y. Akiyama, K. Ito, Roles of homooligomerization and membrane association in ATPase and proteolytic activities of FtsH *in vitro*. *Biochemistry* **40**, 7687-7693 (2001).
178. S. Makino *et al.*, Second transmembrane segment of FtsH plays a role in its proteolytic activity and homo-oligomerization. *FEBS Lett* **460**, 554-558 (1999).
179. Y. Asahara *et al.*, FtsH recognizes proteins with unfolded structure and hydrolyzes the carboxyl side of hydrophobic residues. *J Biochem* **127**, 931-937 (2000).
180. A. Kihara, Y. Akiyama, K. Ito, A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *Embo j* **15**, 6122-6131 (1996).
181. N. Saikawa, Y. Akiyama, K. Ito, FtsH exists as an exceptionally large complex containing HflKC in the plasma membrane of *Escherichia coli*. *Journal of structural biology* **146**, 123-129 (2004).
182. T. Frickey, A. N. Lupas, Phylogenetic analysis of AAA proteins. *J Struct Biol* **146**, 2-10 (2004).
183. A. Kihara, Y. Akiyama, K. Ito, FtsH is required for proteolytic elimination of uncomplexed forms of SecY, an essential protein translocase subunit. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 4532-4536 (1995).
184. Y. Akiyama, A. Kihara, H. Tokuda, K. Ito, FtsH (HflB) is an ATP-dependent protease selectively acting on SecY and some other membrane proteins. *The Journal of biological chemistry* **271**, 31196-31201 (1996).
185. Y. Akiyama, T. Ogura, K. Ito, Involvement of FtsH in protein assembly into and through the membrane. I. Mutations that reduce retention efficiency of a cytoplasmic reporter. *J Biol Chem* **269**, 5218-5224 (1994).
186. C. K. Suzuki *et al.*, ATP-dependent proteases that also chaperone protein biogenesis. *Trends Biochem Sci* **22**, 118-123 (1997).
187. E. van Bloois *et al.*, Detection of cross-links between FtsH, YidC, HflK/C suggests a linked role for these proteins in quality control upon insertion of bacterial inner membrane proteins. *FEBS letters* **582**, 1419-1424 (2008).

188. D. Walker, K. Mosbahi, M. Vankemmelbeke, R. James, C. Kleanthous, The role of electrostatics in colicin nuclease domain translocation into bacterial cells. *The Journal of biological chemistry* **282**, 31389-31397 (2007).
189. L. Mora, K. Moncoq, P. England, J. Oberto, M. de Zamaroczy, The Stable Interaction Between Signal Peptidase LepB of *Escherichia coli* and Nuclease Bacteriocins Promotes Toxin Entry into the Cytoplasm. *The Journal of biological chemistry* **290**, 30783-30796 (2015).
190. M. Chauleau, L. Mora, J. Serba, M. de Zamaroczy, FtsH-dependent processing of RNase colicins D and E3 means that only the cytotoxic domains are imported into the cytoplasm. *The Journal of biological chemistry* **286**, 29397-29407 (2011).
191. E. Alix, A. B. Blanc-Potard, Peptide-assisted degradation of the Salmonella MgtC virulence factor. *The EMBO journal* **27**, 546-557 (2008).
192. N. Thomanek *et al.*, Intricate Crosstalk Between Lipopolysaccharide, Phospholipid and Fatty Acid Metabolism in *Escherichia coli* Modulates Proteolysis of LpxC. *Front Microbiol* **9**, 3285 (2018).
193. C. Katz, E. Z. Ron, Dual role of FtsH in regulating lipopolysaccharide biosynthesis in *Escherichia coli*. *J Bacteriol* **190**, 7117-7122 (2008).
194. C. Herman, D. Thévenet, R. D'Ari, P. Bouloc, Genetics degradation of σ32, the heat shock regulator in *Escherichia coli*, is governed by HflB. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3516-3520 (1995).
195. T. Tomoyasu *et al.*, *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor σ32. *EMBO Journal* **14**, 2551-2560 (1995).
196. S. Langklotz, U. Baumann, F. Narberhaus, Structure and function of the bacterial AAA protease FtsH. *Biochimica et Biophysica Acta - Molecular Cell Research* **1823**, 40-48 (2012).
197. A. Kihara, Y. Akiyama, K. Ito, Host regulation of lysogenic decision in bacteriophage lambda: transmembrane modulation of FtsH (HflB), the cII degrading protease, by HflKC (HflA). *Proc Natl Acad Sci U S A* **94**, 5544-5549 (1997).
198. Y. Shotland *et al.*, Proteolysis of the phage lambda CII regulatory protein by FtsH (HflB) of *Escherichia coli*. *Molecular microbiology* **24**, 1303-1310 (1997).
199. F. Narberhaus, M. Obrist, F. Fuhrer, S. Langklotz, Degradation of cytoplasmic substrates by FtsH, a membrane-anchored protease with many talents. *Research in microbiology* **160**, 652-659 (2009).
200. A. Kihara, Y. Akiyama, K. Ito, Revisiting the lysogenization control of bacteriophage lambda. Identification and characterization of a new host component, HflD. *J Biol Chem* **276**, 13695-13700 (2001).
201. T. Tatsuta, D. M. Joob, R. Calendar, Y. Akiyama, T. Ogura, Evidence for an active role of the DnaK chaperone system in the degradation of sigma(32). *FEBS Lett* **478**, 271-275 (2000).
202. B. Lim *et al.*, Heat shock transcription factor sigma32 co-opts the signal recognition particle to regulate protein homeostasis in *E. coli*. *PLoS Biol* **11**, e1001735 (2013).
203. G. Klein, N. Kobylak, B. Lindner, A. Stupak, S. Raina, Assembly of lipopolysaccharide in *Escherichia coli* requires the essential LapB heat shock protein. *J Biol Chem* **289**, 14829-14853 (2014).
204. S. Mahalakshmi, M. R. Sunayana, L. SaiSree, M. Reddy, yciM is an essential gene required for regulation of lipopolysaccharide synthesis in *Escherichia coli*. *Mol Microbiol* **91**, 145-157 (2014).
205. J. Began *et al.*, Rhomboid intramembrane protease YqgP licenses bacterial membrane protein quality control as adaptor of FtsH AAA protease. *Embo j*, e102935 (2020).
206. D. W. Basta, M. Bergkessel, D. K. Newman, Identification of Fitness Determinants during Energy-Limited Growth Arrest in *Pseudomonas aeruginosa*. *mBio* **8** (2017).
207. D. W. Basta, D. Angeles-Albores, M. A. Spero, J. A. Ciemniecki, D. K. Newman, Heat-shock proteases promote survival of *Pseudomonas aeruginosa* during growth arrest. *Proc Natl Acad Sci U S A* **117**, 4358-4367 (2020).
208. A. Hinz, S. Lee, K. Jacoby, C. Manoil, Membrane proteases and aminoglycoside antibiotic resistance. *Journal of bacteriology* **193**, 4790-4797 (2011).
209. J. Sacharz *et al.*, Sub-cellular location of FtsH proteases in the cyanobacterium *Synechocystis* sp. PCC 6803 suggests localised PSII repair zones in the thylakoid membranes. *Mol Microbiol* **96**, 448-462 (2015).
210. K. Nishimura, Y. Kato, W. Sakamoto, Chloroplast Proteases: Updates on Proteolysis within and across Suborganellar Compartments. *Plant physiology* **171**, 2280-2293 (2016).
211. S. D. Moore, R. T. Sauer, The tmRNA system for translational surveillance and ribosome rescue. *Annu Rev Biochem* **76**, 101-124 (2007).
212. F. Fuhrer, S. Langklotz, F. Narberhaus, The C-terminal end of LpxC is required for degradation by the FtsH protease. *Mol Microbiol* **59**, 1025-1036 (2006).
213. F. Fuhrer *et al.*, Sequence and length recognition of the C-terminal turnover element of LpxC, a soluble substrate of the membrane-bound FtsH protease. *J Mol Biol* **372**, 485-496 (2007).
214. L. M. Bittner, K. Westphal, F. Narberhaus, Conditional Proteolysis of the Membrane Protein YfgM by the FtsH Protease Depends on a Novel N-terminal Degron. *J Biol Chem* **290**, 19367-19378 (2015).

215. D. Bertani, A. B. Oppenheim, F. Narberhaus, An internal region of the RpoH heat shock transcription factor is critical for rapid degradation by the FtsH protease. *FEBS Lett* **493**, 17-20 (2001).
216. K. Westphal, S. Langklotz, N. Thomanek, F. Narberhaus, A trapping approach reveals novel substrates and physiological functions of the essential protease FtsH in *Escherichia coli*. *J Biol Chem* **287**, 42962-42971 (2012).
217. C. Lindemann *et al.*, Next-Generation Trapping of Protease Substrates by Label-Free Proteomics. *Methods Mol Biol* **1841**, 189-206 (2018).
218. J. M. Flynn, S. B. Neher, Y. I. Kim, R. T. Sauer, T. A. Baker, Proteomic discovery of cellular substrates of the ClpXP protease reveals five classes of ClpX-recognition signals. *Mol Cell* **11**, 671-683 (2003).
219. T. T. Hoang, R. R. Karkhoff-Schweizer, A. J. Kutchma, H. P. Schweizer, A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77-86 (1998).
220. G. Ditta, S. Stanfield, D. Corbin, D. R. Helinski, Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti. *Proc Natl Acad Sci U S A* **77**, 7347-7351 (1980).
221. K. H. Choi, H. P. Schweizer, mini-Tn7 insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa*. *Nat Protoc* **1**, 153-161 (2006).
222. M. Boehm *et al.*, Subunit organization of a synechocystis hetero-oligomeric thylakoid FtsH complex involved in photosystem II repair. *Plant Cell* **24**, 3669-3683 (2012).
223. V. Krynicka *et al.*, Two essential FtsH proteases control the level of the Fur repressor during iron deficiency in the cyanobacterium *Synechocystis* sp. PCC 6803. *Mol Microbiol* **94**, 609-624 (2014).
224. J. Arends, N. Thomanek, K. Kuhlmann, K. Marcus, F. Narberhaus, *In vivo* trapping of FtsH substrates by label-free quantitative proteomics. *Proteomics* **16**, 3161-3172 (2016).
225. M. Garcia-Lorenzo, A. Zelisko, G. Jackowski, C. Funk, Degradation of the main Photosystem II light-harvesting complex. *Photochem Photobiol Sci* **4**, 1065-1071 (2005).
226. D. Yao *et al.*, Localization of the small CAB-like proteins in photosystem II. *J Biol Chem* **282**, 267-276 (2007).
227. S. A. Bissonnette, I. Rivera-Rivera, R. T. Sauer, T. A. Baker, The IbpA and IbpB small heat-shock proteins are substrates of the AAA+ Lon protease. *Mol Microbiol* **75**, 1539-1549 (2010).
228. N. H. Bhat, R. H. Vass, P. R. Stoddard, D. K. Shin, P. Chien, Identification of ClpP substrates in Caulobacter crescentus reveals a role for regulated proteolysis in bacterial development. *Mol Microbiol* **88**, 1083-1092 (2013).
229. R. C. Bruckner, P. L. Gunyuzlu, R. L. Stein, Coupled kinetics of ATP and peptide hydrolysis by *Escherichia coli* FtsH protease. *Biochemistry* **42**, 10843-10852 (2003).
230. S. B. Hari, R. T. Sauer, The AAA+ FtsH Protease Degrades an ssrA-Tagged Model Protein in the Inner Membrane of *Escherichia coli*. *Biochemistry* **55**, 5649-5652 (2016).
231. T. Ogura *et al.*, Structure and function of the *ftsH* gene in *Escherichia coli*. *Research in Microbiology* **142**, 279-282 (1991).
232. C. Y. Chu *et al.*, Function of the Borrelia burgdorferi FtsH Homolog Is Essential for Viability both *In Vitro* and *In Vivo* and Independent of HflK/C. *mBio* **7**, e00404-00416 (2016).
233. S. Langklotz, M. Schäkermann, F. Narberhaus, Control of lipopolysaccharide biosynthesis by FtsH-mediated proteolysis of LpxC is conserved in enterobacteria but not in all gram-negative bacteria. *Journal of Bacteriology* **193**, 1090-1097 (2011).
234. M. A. Jacobs *et al.*, Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **100**, 14339-14344 (2003).
235. Y. Akiyama, A. Kihara, K. Ito, Subunit a of proton ATPase F0 sector is a substrate of the FtsH protease in *Escherichia coli*. *FEBS Lett* **399**, 26-28 (1996).
236. A. De Soyza *et al.*, Developing an international *Pseudomonas aeruginosa* reference panel. *Microbiologyopen* **2**, 1010-1023 (2013).
237. K. Amberg-Johnson *et al.*, Small molecule inhibition of apicomplexan FtsH1 disrupts plastid biogenesis in human pathogens. *Elife* **6** (2017).