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DISSERTATION

Titel der Dissertation

Role of Clp-mediated proteolysis in the Gram-positive human pathogen *Streptococcus pyogenes*

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Contents

Abstract

During infection, pathogenic bacteria are exposed to hostile conditions such as acidic pH, increase of temperature, starvation or oxidative stress leading to an accumulation of aggregated, unfolded or denatured proteins, which are toxic for the cell. By degrading or refolding the damaged proteins, the Clp protein family allows the maintainance of protein homeostasis in bacterial cells. Moreover, previous studies showed that the Clp proteins are involved in the virulence of Gram-positive bacteria such as *Staphylococcus aureus, Listeria monocytogenes* and *Streptococcus pneumoniae* but the mechanisms by which this class of proteins regulates virulence factor expression remain unclear. Our study focused on the impact of the Clp-mediated proteolysis in the severe human pathogen *Streptococcus pyogenes*. To investigate Clp protein functions, in-frame deletion mutants of the protease encoding gene *clpP* and the Clp ATPase encoding genes *clpC*, *clpE* and *clpX* were generated and compared to the wild-type strain in a broad phenotypic analysis. We show that Δ*clpX* and Δ*clpP* mutants display aberrant phenotypes including a delay of growth, increased biofilm and aggregate formation, altered chain formation and cell morphology, deficiency in transformation and increased adhesion to epithelial cells. Additionally, a lack of *clpX* or *clpP* expression resulted in increased tolerance to heat and antibiotic stress while the sensitivity to oxidative and osmotic stress was elevated. At the molecular level, we demonstrate that ClpX and ClpP but not ClpE influence the expression of exoproteins, some of which were identified by mass spectrometry. Both ClpX and ClpP positively regulate the expression of cysteine protease SpeB whereas they negatively control the expression of M protein, the streptococcal inhibitor of complement (Sic) and streptokinase (Ska). Transcription of the virulence gene regulators, RopB, CovR, FasA and *fasX* RNA, was also affected in the Δ*clpX* and Δ*clpP* mutants. We propose a model where the ClpXP-mediated proteolysis is required for the transcriptional regulation of RopB, the main regulator for the entry into stationary phase, thereby altering the physiology, adaptation and virulence of *S. pyogenes*.

Zusammenfassung

Während einer Infektion sind pathogene Bakterien unterschiedlichen lebensfeindlichen Einflüssen wie saurem pH, erhöhter Temperatur, Nährstoffknappheit oder oxidativem Stress ausgesetzt, was zur Anhäufung von defekten, entfalteten oder denaturierten Proteinen führt, die für die Zellen toxisch sind. Die Familie der Clp Proteine ist für den Abbau von diesen aggregierten Proteinen und damit die Aufrechterhaltung des Protein Gleichgewichts verantwortlich. Frühere Studien haben gezeigt, dass Clp Proteine auch an der Virulenz von gram positiven Bakterien wie *Staphylococcus areus*, *Listeria monocytogenes* und *Streptococcus pneumoniae* beteiligt sind, wobei der dafür verantworliche Mechanismus bislang unbekannt ist. Unsere Untersuchungen beschäftigen sich besonders mit dem Einfluss der Clp vermittelten Proteolyse auf das human pathogene Bakterium Streptococcus pyogenes. Zu diesem Zweck wurden „in-frame“ Deletionsmutanten der Gene ClpP, kodierend für eine Protease sowie der Gene für die Clp ATPasen ClpC, ClpX und ClpE hergestellt und in unterschiedlichen phänotypischen Untersuchungen mit einem wildtyp Stamm verglichen. Wir konnten zeigen, dass diese *ΔclpX* und *ΔclpP* Mutanten phänotypische Veränderungen aufweisen hinsichtlich einer zeitlichen Wachstumsverzögerung, einer verstärkten Bildung von Biofilmen und Aggregaten, einer veränderten Kolonieform und Morphologie, einer reduzierten Transformierbarkeit als auch einer erhöhten Adhesion an Epithelzellen. Weiters führt das Fehlen von ClpX und ClpP zu einer höheren Toleranz gegenüber Temperaturveränderung und antibiotischen Substanzen wohingegen die Sensitivität in Bezug auf oxidativen oder osmotischen Stress zunimmt. Zudem konnten wir feststellen, dass ClpX, ClpP aber nicht ClpE die Expression von Exoproteinen beeinflussen, die zum Teil mittels Massenspektrometrie identifiziert wurden. Zusätzlich verstärken ClpX und ClpP die Expression der Zystein Protease SpeB, während die Expression des M Proteins, des Komplement-Inhibitors (Sic) sowie der Streptokinase (Ska) reduziert wird. Ausserdem beeinflussen ClpX und ClpP auch die Virulenz Regulatoren RopB, CovR, FasA und fasX RNA. Daher postulieren wir ein Model bei dem die ClpXP vermittelte Proteolyse von entscheidender Bedeutung für die transkriptionelle Regulierung von RopB, einem wichtigen Regulator für den Eintritt in die stationäre Phase, ist und dadurch die Physiologie, Adaption und Virulenz von *S. pyogenes* beeinflusst wird.

Abbreviations

°C: degree Celsius
aa: amino acids
DNA: desoxyribonucleic acid
RNA: ribonucleic acid
mRNA: messenger RNA
rRNA: ribosomal RNA
tRNA: transfert RNA
ATP: adenosine tri-phosphate
bp: base paire
cfu: colony forming unit
CO_2: carbon dioxide
kDa: kilo-Dalton
dH_2O: distilled water
ddH_2O: double distilled water
FCS: foetal calf serum
h: hour
H_2O_2: hydrogen peroxide
LB: Luria broth
M: molar
min: minute
mg: milli-gram
ml: milli-liter
mM: milli-molar
mg: milli-gram
ml: milli-liter
mM: milli-molar
LB: Luria broth
M: molar
min: minute
mg: milli-gram
ml: milli-liter
mM: milli-molar
nm: nano-meter
nt: nucleotide
O_2: oxygen
OD: optique density
PBS: phosphate buffer saline
rpm: rotation per minute
sec: seconde
C-terminal: carboxy terminal end
N-terminal: amino terminal end
µg: micro-gram
µl: micro-liter
Xgal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Introduction

1. Proteolysis generalities

Proteolysis was originally thought to be a processus only involved in the metabolism of the cells, to recycle proteins and generate amino acids and energy [1]. Later on, proteolysis was found to be a key component in stress response by degrading biologically inactive aggregates, which interfere with vital cell functions. Furthermore, proteolysis is involved in the regulation of various biological processes including cell division, physiology and pathogenicity by controlling the stability of key regulators and thus promoting a rapid response to environmental changes. Proteolysis is required as a regulatory mechanism of protein expression either by generating specific cleavage of domains or motifs of proteins, changing their conformation or by complete degradation of proteins [2]. Active and regulated protein degradation is essential in all living cells and is conserved from bacteria to eukaryotic organisms. However, the energy cost necessary to make and degrade proteins is massive, so is proteolysis really beneficial for the cell? Indeed, this regulation allows extremely fast kinetics required to adapt quickly to new environments [2-4].

In eukaryotes, ATP-dependent proteolysis is carried out by the 26S proteasome whereas in bacterial cells degradation is performed by several ATP-dependent proteases such as FtsH, Lon and caseinolytic protease (Clp). These proteases, including the eukaryotic 26S, do not show a high sequence identity but share common properties [5]. They are located in the cytoplasm, depend on ATP and degrade proteins into 10-15 amino acids peptides. Their ATPase and protease activities are carried out by separated domains where the ATPase domain blocks the entrance to the proteolytic site. The substrate degradation begins with the binding of the substrate to the ATPase domain which unfolds the protein and translocates it into the proteolytic domain for degradation. FtsH and Lon exhibit both domains on the same protein whereas the Clp proteolytic complex is a two-component protease where protease and ATPase activities are carried out by two distinct proteins. In addition, FtsH possesses two trans-membrane domains which also enable the degradation of membrane-associated proteins [6]. All these ATP-dependent proteases are located in the cytoplasm. Substrate recognition is the most important step of proteolysis. It needs to be tightly regulated to avoid every cytosolic protein to be degraded at all time. Hydrophobic motifs and degradation tags exhibited by the substrate have been identified to specically direct proteins for degradation [7].

2. Clp proteolysis

The Clp proteolytic complex is composed of a two-component cylindrical architecture with a chaperone adenosine triphosphatase (ATPase: ClpA, ClpC, ClpE, ClpX and ClpY) responsible for the specific recognition and unfolding of substrates and a protease (ClpP and ClpQ) involved in the degradation of polypeptides. To avoid unintentional degradation of proteins, the proteolytic site of the protease is located deep inside the barrel structure and can only be reached through a really narrow pore. For this purpose, the substrate needs to be unfolded to access the protease and be degraded. The Clp ATPase use the energy produced by the hydrolysis of ATP to unfold the substrate and translocate it into the protease barrel [8-9]. Clp proteins are ubiquitous in prokaryotes but also present in mitochondria and chloroplasts of eukaryotic cells. The various combinations of Clp proteases and Clp ATPases result in an inventory of several Clp proteolytic complexes. The protease ClpP interacts with ClpA, ClpC, ClpE and ClpX whereas ClpQ interacts exclusively with ClpY. ATPases interact as well with adaptor proteins to enhance the recognition specificity of the substrate and its delivery into the protease barrel. The proteolytic activity of the protease is not sequence specific therefore the specific recognition of the substrate is carried out by Clp ATPases and adaptor proteins. This section of the manuscript will focus on the particular characteristics of the different Clp protease components.

2.1. Clp ATPases

Clp ATPases or HSP100 are members of the AAA^+ family (ATPases associated with various cellular activities) that is present in all kingdoms and known to harbor a highly conserved AAA+ domain involved in the hydrolysis of ATP. The main role of AAA+ proteins is to induce conformational changes into target proteins thus promoting their unfolding. Thus, AAA+ proteins are involved in proteolysis, disassembly of protein aggregates, activation of transcription, maintenance of organelle functions or chromosome replication [10]. The AAA+ domain (200-250 amino acids) contains several motifs that distinguish AAA+ proteins from other classical ATPases. First, the classic P-loop NTPase, containing the Walker A and Walker B motifs is involved in the interaction and hydrolysis of nucleotides (Fig. 1). The Walker A motif participates in the binding to the phosphate of ATP molecules. Mutations in the Walker A domain inhibit nucleotide binding therefore inactivating AAA+ proteins. On the other hand, the Walker B motif also participates in the

binding of the nucleotide but is in addition responsible for the hydrolysis of ATP. Mutations in the Walker B domain allow the binding but not the hydrolysis of ATP. As ATP is required for the interaction with AAA+ substrates, these Walker B mutant proteins bind the substrates without releasing them and thus can be used as substrate traps [11]. The Sensor 1 and 2 domains interact with the Walker B to hydrolyze ATP [12-13]. The arginine finger domain is involved in the oligomerisation of AAA+ proteins into hexameric rings (Fig. 1) [14].

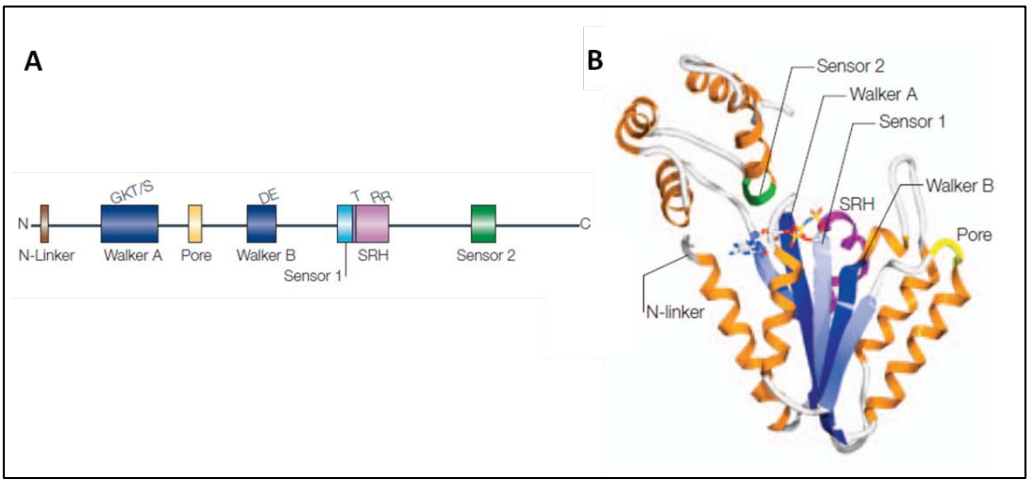

Fig. 1: Organisation of the AAA+ domain. (A) Linear diagram of the key elements of the AAA+ domain. The letters on top of the elements represent the amino acids required for binding and hydrolysis of ATP. (B) Structure of the AAA+ domain showing the approximate position of the various motifs. The position of the nucleotide is represented by a stick AMP molecule. Adapted from [15].

Based on their number of AAA+ domains, Clp ATPases are divided in two classes: the class I, composed of ClpA, ClpB, ClpC, ClpE and ClpL, contains two consecutive AAA+ domains separated by a linker region of variable length whereas the class II, composed of ClpX and ClpY, exhibits only one AAA+ domain (Fig. 2). The functional significance of having one versus two AAA+ domains remains unknown. In addition to the AAA+ domain, Clp ATPases possess an amino-terminal region involved in chaperone oligomerisation, formation of the proteolytic machine and in the binding of adaptors and substrates [16-18]. This N-terminal region differs between the Clp proteins. For example, for ClpA, ClpB, and ClpC it consists of two homologous repeats of 75 amino acids while ClpX and ClpE exhibit a Zn-binding domain. Morever, ClpA, ClpC, ClpE and ClpX can interact with ClpP thanks to the presence of a ClpP recognition domain at their C-terminal end known as the P-loop, exhibiting a conserved isoleucine-guanine-phenylalanine (IGF) motif [19]. However, ClpB

and ClpL lack this P-loop and can only act as chaperones. Interestingly, when the P-loop from ClpA or ClpX is added to ClpB, the substrate can be delivered to ClpP for degration [20].

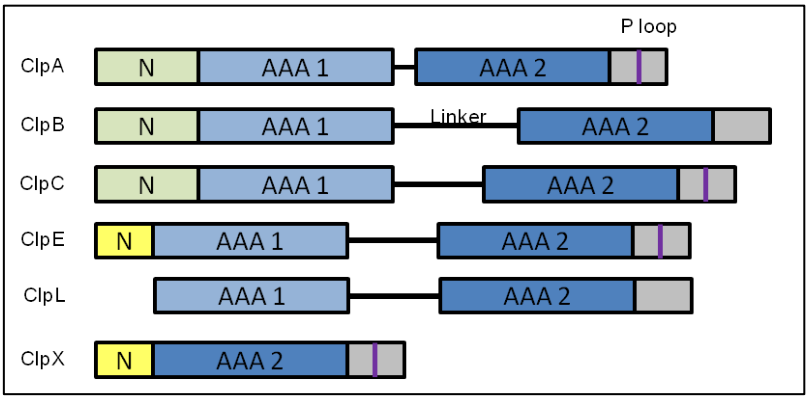

Fig. 2: Domain organisation of Clp ATPases. The AAA+ domain is pictured in blue, the amino-terminal domain is in green and the zinc binding finger is in yellow. The P-loop required for the binding to ClpP is represented in purple.

ClpA and ClpC are orthologues and bacteria exhibit one or the other. ClpA is found in Gram-negative bacteria whereas ClpC is present in Gram-positive bacteria and cyanobacteria. ClpY and its associated protease ClpQ are most often present alongside ClpA in Gram-negative bacteria but can be found as well in some Gram-positive bacteria such as *S. aureus.* ClpX is the most ubiquitous Clp ATPase and is present in almost all bacteria (Table 1).

To be active, Clp ATPases as the other AAA+ proteins, assemble into homohexameric rings with ATP binding sites located at the interface between monomers and a central pore necessary for the translocation of substrates (Fig. 3A). The binding of ATP promotes conformational changes within the hexamer enabling the binding of the substrate to the amino-terminal domain and stabilization of the complex [21-23]. Clp ATPases can bind the protease via their surface exposed P-loop only when bound to the substrate, in a hexameric ring conformation and activated [24-25]. In the case of ClpC in *Bacillus subtilis*, it was shown that its oligomerisation requires the interaction with an adaptor protein such as MecA [18]. Thus, the activity of ClpC depends on the presence of this adaptor proteins acting as regulator of ClpC-mediated proteolysis.

Besides acting with proteases, Clp ATPases or Hsp100 act as chaperones to eliminate stress induced protein aggregates (Fig. 3B). The mechanism of disaggregation is based on the same principle: forcing the unfolding by translocation of the substrate peptide through the central pore of the chaperone. In this case, ClpB unfolds single peptides from aggregates and

cooperates with other chaperones such as the Hsp70 system to properly refold proteins [20]. Furthermore, Clp chaperones can also unfold stable proteins to disassemble protein complexes. For example, ClpX was found to unfold MuA subunits via their C-terminal end leading to the destabilization of MuA tetramers, a bacterial virus inhibiting DNA replication [26]. Another example is ClpA, which converts the plasmid replication initiator RepA from an inactive dimer to an active monomer [27].

	E. coli	*B. subtilis*	*L. monocytogenes*	*S. aureus*	*S. pneumoniae*	*S. mutans*	*S. pyogenes*
ATPases	ClpA ClpX ClpY ClpB	ClpC ClpX ClpE ClpY	ClpC ClpX ClpE ClpB	ClpC ClpX ClpY ClpB ClpL	ClpC ClpX ClpE ClpL	ClpC ClpX ClpE ClpB ClpL	ClpC ClpX ClpE ClpB ClpL
Proteases	ClpP ClpQ FtsH LonA LonB	ClpP ClpQ FtsH	ClpP1/ClpP2 FtsH	ClpP ClpQ FtsH	ClpP FtsH	ClpP FtsH	ClpP FtsH
Regulation		CtsR McsA McsB MecA	CtsR McsA McsB MecA	CtsR McsA McsB MecA	CtsR MecA	CtsR MecA	CtsR MecA

Table 1: Clp proteins present in *E. coli* and Gram-positive bacteria.

2.2. Clp protease

ClpP is a serine protease, exhibiting a conserved catalytic triad (serine-histidine-aspartic acid), that assembles into a double heptameric ring structure forming a barrel with a 1-2 nm pore where the proteolytic active sites are protected (Fig. 3A) [28]. The ClpP protease pore is narrow and only peptides smaller than 5 amino acids can enter the pore without the need for a chaperone. In fact, the entry of larger peptides is blocked by a loop present at the amino-terminal region of ClpP. In order to degrade larger peptides, the ClpP complex binds on both sides of the barrel to a Clp ATPase hexameric ring which unfolds and translocates the substrate into the protease barrel for degradation. ClpP interacts via a surface hydrophobic patch leading to hydrophobic interaction with residues of the P loop of the ATPase [29-30]. In addition, the N-terminal domain of ClpP is essential for the binding of the chaperone to the protease [31]. The conformational changes triggered upon interaction with the ATPase ring open the pore for larger substrates [30, 32]. ClpP degrades proteins in a processive manner, producing smaller peptides of approximately 15 amino acids.

Several studies using fluorescently labelled proteins investigated the localization of ClpCP, ClpEP and ClpXP proteolytic complexes in *B. subtilis* [33-35]. They revealed that these complexes gather at the vicinity of the cell poles. Additional reports described that adaptor proteins including MecA and McsB also gather with the proteolytic complexes at the poles [33]. This suggests that in *B. subtilis*, Clp-mediated proteolysis is localized at the poles where most of the protein synthesis and protein quality control take place [36-37].

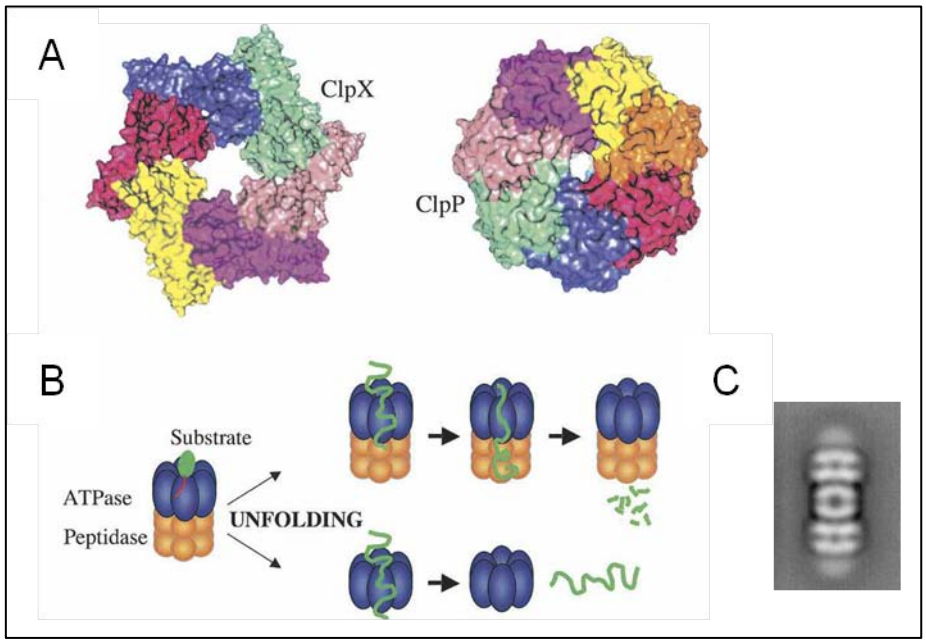

Fig. 3: Clp proteolytic complex. (A) Structure of ClpX hexameric ring and ClpP heptameric barrel [38]. (B) Pathways involving Clp proteins: refolding or degradation. Clp ATPase is in blue and Clp protease is in orange. (C) Electron microscopy picture of the ClpXP proteolytic complex [39].

2.3. Adaptor proteins

In order to enhance and diversify their substrate spectrum, the Clp proteolytic complexes interact with specific recognition factors also called adaptor proteins [40]. These adaptors can be specific for one protein or a whole group of substrates. In some cases, they are essential for the recruitment of the substrate whereas in other cases they only promote the substrate recognition and/or activate the chaperone. First discovered in *B. subtilis*, adaptor proteins have been identified to interact and modulate the activity of ClpA, ClpC and ClpX in *B. subtilis* and *E. coli* (Table 2). However, no adaptor proteins have been found for ClpE so far. Unfortunately, these adaptor proteins are unrelated in sequence as well as in structure, which makes difficult the identification of new adaptors by bioinformatic predictions.

Most importantly, adaptor proteins provide an additional control level in the tightly regulated process of proteolysis. Regulation by adaptors is an efficient strategy to quickly and specifically respond to environmental changes. For instance, their affinity for the substrate can be controlled by modifications as demonstrated by RssB, an adaptor which only transfers the substrate σ^S to ClpXP when phosphorylated. Alternatively, other proteins can compete with the substrate to bind the adaptor therefore inhibiting the interaction between the substrate and the protease. This is illustrated by the binding of ComS to the adaptor MecA releasing the substrate ComK, a regulator of competence in *B. subtilis*. The role and regulation of adaptor proteins in combination with their substrates will be discussed below (Table 2).

2.4. Substrates

2.4.1. SsrA-tagged proteins

The SsrA tag consists of 11 amino acids added to the carboxy-terminal end of nascent peptide chains blocked in the ribosome [41]. *ssrA* is a small RNA acting as tRNA and mRNA, that enters in the A-site of the ribosome stalled at the 3' end of a truncated mRNA. When stalled, the ribosome switches template to the *ssrA* RNA thus adding 11 amino acids to the carboxy-terminal end of the peptide. This tag targets these truncated proteins to degradation by proteases such as the ClpAP and ClpXP complexes. In the case of the ClpXP proteolytic complex, the mediated degradation can proceed without the help of an adaptor. However, interaction with SspB increases the concentration of the substrate in the vicinity of the protease therefore stimulating its degradation [42-45]. Indeed, SspB forms a dimer that binds the SsrA tag via its N-terminal domain and docks to ClpX through a C-terminal binding motif. In *E. coli*, the SsrA tagged protein can also be degraded by the ClpAP complex [42]. *In vivo,* the ClpAP complex interacts with the adaptor ClpS, which inhibits the degradation of the SsrA tagged proteins but enhances degradation of other ClpAP substrates [46-47]. Nevertheless, when the ClpXP complex is overwhelmed, the ClpAP protease can degrade *in vivo* SsrA tagged substrates.

ATPase	Adaptor	Substrate	Reference
ClpA	ClpS	Misfolded and aggregated proteins	[40]
		N-end rule protein	[47]
	no adaptor	SsrA tag	[40]
ClpX	SspB	SsrA tag	[42-43, 45]
		RseA	[45]
	RssB	Sigma S	[48]
	UmuD	UmuD'	[49]
	no adaptor	RsiW	[50]
		LexA fragments	[51]
	unknown	Dps	[52-53]
	unknown	Sda	[54]
	YjbH	Spx	[55-56]
ClpC	MecA	ComK	[57]
		ComS	[57-58]
		DegU	[59]
		Spx	[60]
		Misfolded and aggregated proteins	[61]
	YpbH	Misfolded and aggregated proteins	[61]
		Spx (in vitro)	[60]
	McsB	CtsR	[18]
	unknown	SpoIIAB	[62]
	unknown	MurAA	[63]

Table 2: Adaptor proteins and substrates identified for Clp proteins in *E. coli* (white) and *B. subtilis* (green).

2.4.2. Sigma S

RpoS or σ^S is the main regulator of stress response in *E. coli*. Its expression is activated under various stress conditions including starvation, pH shift or heat-shock [64]. σ^S is specifically degraded by the ClpXP complex in association with the adaptor protein RssB [65]. The phosphorylated RssB, which exhibits higher affinity to σ^S than its unphosphorylated form, targets σ^S to degradation via the ClpXP complex. Following degradation, RssB is released from the proteolytic complex in its dephosphorylated form. Therefore, the stability of σ^S depends on the phosphorylation of RssB, which belongs to a two-component system where ArcB, the sensor kinase is responsible for its phosphorylation. Upon stress, the activation of ArcB is reduced, leading to a decreased phosphorylation of RssB and thus to stabilization of σ^S [66]. Furthermore, Ira proteins have been identified to interfere with RssB adaptor function therefore stabilizing σ^S during specific stress conditions. In response to phosphate starvation, IraP binds to RssB thus releasing σ^S [67]. On the other hand, upon Mg2+ or Ca2+ starvation IraM binds RssB, whereas IraD binds RssB following DNA damages [68-69]. Although the

Ira proteins bind RssB, no specific binding motif or sequence similarities have been identified. Therefore it is difficult to identify other proteins involved in RpoS regulation [68].

2.4.3. Spx

In *B. subtilis*, Spx was first identified as a suppressor locus of *clpP* and *clpX* mutations. Spx is a global transcriptional regulator that exerts both positive and negative control on the expression of multiple genes during oxidative stress [70-71]. Disulfide stress leads to induction of the Spx regulon, which includes *trxA* encoding for a thioredoxin, *trxB* coding for a thioredoxin reductase and other genes involved in the oxidative stress response [72]. Spx-dependent regulation of gene expression requires interaction with RNA polymerase holoenzyme [73-74]. Spx exhibits a motif similar to the SsrA tag on its C-terminal end, which promotes its degradation by the ClpXP complex. Later on, YjbH was identified to be the first adaptor protein of the ClpXP complex in *B. subtilis* and exhibits as ClpX a zinc binding domain. YjbH interacts specifically with Spx inducing a conformational change that leads to the exposure of the C-terminal motif of Spx therefore promoting its degradation by ClpXP [55]. Upon oxidative stress, the zinc atoms dissociate from ClpX and YjbH resulting in the loss of ATPase activity of ClpX, the release of Spx from YjbD and the activation of Spx-mediated oxidative stress response [55, 75].

2.4.4. ComK / ComS: regulation of competence

ComK, the main regulator of competence in *B. subtilis,* activates the expression of DNA-uptake components [76-77]. In non-competent cells, inactivation of ComK involves its degradation by ClpCP after binding to the adaptor protein MecA [57]. The binding of MecA to the ClpC monomers is required to promote the oligomerisation of ClpC and activates the ClpCP-MecA complex [18]. During stationary phase, competence is activated by quorum sensing signals, leading to the expression of ComS [78]. ComS and ComK share a motif required for the binding to MecA and therefore compete for MecA-dependent degradation by the ClpCP protease [58]. Consequently, under conditions where ComS is expressed, it is degraded by ClpCP-MecA instead of ComK, which escapes its degradation and can thus activate its expression as well as the expression of genes required for competence [57]. Unfortunately, the MecA-binding motif in ComK and ComS was not found in any other proteins of *B. subtilis* thus suggesting that this motif is specific for the regulation of competence by ClpCP-MecA. However, in contrast to ComK, MecA orthologues are found in

most Gram-positive bacteria expressing ClpC, thus suggesting that MecA could be involved in other processes in Gram-positive bacteria [58, 61].

YpbH, a paralogue of MecA in *B. subtilis*, acts as well as an adaptor for ClpC but is not involved in the regulation of ComK [61]. MecA and YpbH promote the degradation of misfolded and aggregated proteins by the ClpCP complex [61]. Interestingly, ClpX is also involved in the regulation of competence in *B. subtilis*. In fact, ClpX and ClpP are required for the expression of *comS* probably by degrading a repressor of *comS* transcription [79].

2.4.5. CtsR: heat-shock induced regulation of Clp proteins

In the Gram-positive model organism *B. subtilis*, expression of heat-shock genes involves at least four different regulatory mechanisms [80-81]. Class I heat-shock genes encode classical chaperones, such as GroES, GroEL and DnaK, and are controlled by the HrcA repressor. Class II genes encode general stress proteins whose transcription is dependent on the alternative sigma factor σ^B [81]. Class III heat-shock genes encoding ClpP and two Hsp100 Clp ATPases, ClpC and ClpE, form part of the CtsR stress response regulon. Finally, class IV includes stress response genes whose expression is independent of HrcA, σ^B or CtsR (such as FtsH, LonA, ClpX) and whose regulatory mechanisms remain to be identified [80-82]. Expression of the class III genes is dependent on the negative regulator of transcription, CtsR. The *ctsR* gene is the first gene of the *clpC* operon, which comprises the kinase activator encoding gene *mcsA*, an arginine kinase encoding gene *mcsB* and the ATPase encoding gene, *clpC* [83-85]. CtsR binds as a dimer to a tandem heptanucleotide repeat A/GGTCAAA/T located in the promoter region of the *clpP* and *clpE* genes and of the *clpC* operon, indicating that CtsR inhibits as well its own expression [80, 82, 86-87]. Morever, in *B. subtilis*, *clpE* appears to be the gene the most tightly repressed by CtsR as five binding sites are located in its promoter region [82]. Interestingly, the binding motif recognized by CtsR was found intact in the promoter regions of *clpP, clpE* and the *clpC* operon of other Gram-positive bacteria including *S. pneumoniae* and *S. pyogenes*, which also possess a *ctsR* homologue. This indicates that the heat-shock regulation mediated by CtsR is highly conserved in Gram-positive bacteria [80]. Surprisingly, the CtsR binding motifs were also found in promoters of chaperone GroESL and DnaK encoding genes in *S. pneumoniae, S. mutans, S. aureus, S. pyogenes* and *L. monocytogenes*, indicating that in these organisms, these chaperones can be regulated by both HcrA and CtsR [88-91]. Notably, the promoter

region of *clpX* does not exhibit any CtsR binding motif in any organism examined so far, thus suggesting that *clpX* is not a member of the CtsR regulon [92].

Regulation of the negative regulator CtsR is a key component of the heat-shock response. ClpC and the arginine kinase McsB are activators of this regulation by promoting the degradation of CtsR by the ClpP protease [93-94]. Under normal growth conditions, MscB is inhibited by its interaction with ClpC. Upon stress conditions, ClpC interacts preferentially with MecA to degrade stress induced misfolded proteins, while releasing MscB. Free McsB, activated by McsA, inhibits CtsR by phosphorylating arginine residues located close to its DNA binding site therefore inhibiting its dimerisation [94-95]. Phosphorylated monomers of CtsR are unable to interact with promoters therefore inducing expression of the class III heat-shock genes. Moreover, the phosphorylated McsB acts as an adaptor protein of ClpC that targets CtsR for degradation by the ClpCP complex [96]. In this context, the ClpC-MecA complex acts as a sensor of misfolded proteins resulting from stress which leads to lifting the inhibition on heat-shock genes. Finally, the phosphatase YwlE was shown to dephoshoprylate McsB and CtsR *in vitro*, suggesting a putative switch-off mechanism of the class III heat-shock response [95].

2.5. Role of Clp proteins in bacterial virulence

In non stressed cells, an increase in global protein degradation is observed when cells reach stationary phase. ClpP appears to be the major determinant of this protein turn-over as cells lacking ClpP show an increase in protein aggregates and do not exhibit a growth phase-dependent regulation of degradation [63]. ClpP was shown to be important during stress response as most *clpP*-deficient strains are restricted in growth upon various stress conditions in Gram-positive bacteria [91, 97-101]. Studies using deficient stains showed that the associated Clp ATPase ClpC, ClpE and ClpX are taking part in the stress induced ClpP-mediated proteolysis in Gram-positive bacteria. The entry of bacterial pathogens into host organisms constitutes dramatic environmental changes enhancing expression of stress response proteins. Their degradation may confer a selective advantage to facultative intracellular bacteria for survival in phagocytes, leading to their proliferation and dissemination throughout the host. There is growing evidence suggesting that Clp proteins play an important role in the survival and virulence of pathogens during host infection. Table 3 regroups the functions of Clp proteins in cell physiology, stress response and regulation of

virulence in the main Gram-positive pathogens *L. monocytogenes*, *S. aureus*, *S. pneumoniae* and *S. mutans*. Some of these functions will be discussed in more detail.

2.5.1. Physiology

ClpP is required to remove misfolded proteins and aggregates from the cytosol. Interestingly the various *clpP*-deficient strains studied so far in *S. aureus, L. monocytogenes*, *S. pneumoniae* and *S. mutans* exhibit a delay of growth when cultivated at normal temperature, most likely due to an accumulation of non-native proteins [87, 99, 101-102]. Therefore, the proteolytic activity of ClpP is necessary for the physiological growth of Gram-positive bacteria. In addition, Clp proteins have been linked to cell division. In fact, in *S. pneumoniae*, *clpC*-deficient strains grow in longer chains compared to wild-type whereas in *L. monocytogenes*, *clpC* and *clpE* mutants show no phenotype concerning cell division [103-104]. However, in the double mutant *clpE-clpC*, cells were elongated and no septum was observed indicating a failure in cell division and suggesting that ClpE acts synergistically with ClpC to promote cell division [104]. Similar phenotypes were observed for the *clpX* and *clpP* mutants in *S. mutans* [105]. The acquisition of foreign DNA is a crucial step in the survival under stress conditions. The role of Clp proteins in the regulation of competence has been analyzed in *B. subtilis* with the degradation of ComK/ComS [57]. In other naturally competent bacteria such as *S. pneumoniae* or *S. mutans*, deletion of *clpP* was associated with a decrease in competence [88, 91]. Furthermore, several studies have reported the impact of Clp proteins in biofilm formation, a well known mechanism to evade the immune response and antibiotic treatment. However, the impact of Clp proteins in this phenotype differs depending on the bacterial species. In the high biofilm producing organism *S. aureus,* the ATPases ClpC and ClpX promote biofilm formation whereas the protease ClpP represses it [83]. In *S. mutans*, biofilm formation is inhibited by ClpX and ClpP while it is enhanced by ClpE [105]. These results show that biofilm formation is repressed by Clp-mediated proteolysis and enhanced by the chaperone activity of some Clp proteins. Notably, ClpX appears to be an essential gene in *S. pneumoniae* as no *clpX*-deficient mutant strain was ever obtained [106]. Interestingly, the *clpP*-deficient mutant strain is viable in *S. pneumoniae* indicating that the chaperone role of ClpX might be essential in this organism.

Table 3: Involvement of the Clp proteins in morphology, stress responses and virulence of Gram-positive pathogens *L. monocytogenes*, *S. aureus*, *S. pneumoniae* and *S. mutans.* The third column indicates the regulation of Clp proteins by CtsR (*).

	Protein	*	Functions of Clp proteins	References
L. monocytogenes	ClpP	+	Growth Resistance to heat and osmolarity stress Virulence in mouse; escape of phagosome and intracellular replication in macrophages and hepatocytes; activation of Listeriolysin O (LLO); inhibition of *sip* and *svpA*; recruitment of actin	[99, 107-110]
	ClpC	+	Cell division Resistance to heat-shock and osmolarity stress Survival in spleen and liver; adhesion and invasion of hepatocytes by activating expression of adhesins; survival in macrophages by promoting escape from the phagosome; inhibition of *sip* and *svpA*	[85, 104, 107-108, 110-111]
	ClpE	+	Cell division Heat tolerance Survival in spleen and liver	[104]
	ClpB	+	Resistance to suboptimal temperature; thermotolerance Virulence in mouse	[90, 112]
S. aureus	ClpP	+	Growth at 37°C; morphology; inhibition of autolysis; inhibition of biofilm formation Tolerance to oxidative stress, osmotic stress, heat-shock, suboptimal temperature Virulence in skin abcess model; inhibition of internalisation; intracellular replication; haemolysin expression derived from *agr* activation; degradation of antitoxin	[83, 101, 113-115]
	ClpC	+	Biofilm formation; long term survival; programmed cell death; TCA cycle Tolerance to heat-shock, oxidative stress Degradation of antitoxin	[83, 113, 116]
	ClpX	-	Biofilm formation Tolerance to oxidative stress, osmotic stress, suboptimal temperature Virulence in bacteraemia and skin abcess model; intracellular replication; haemolysin and PrtA expression derived from *agr* activation; activation of *spa* and *rot* expression	[83, 101, 114, 117-118]
	ClpB	-	Tolerance to heat-shock and thermotolerance Intracellular replication	[83]
	ClpL	-	Tolerance to heat-shock and thermotolerance	[83]
	ClpY	-	Tolerance to heat-shock	[119]
	ClpQ	-	Tolerance to heat-shock	[119]
S. pneumoniae	ClpP	+	Inhibition and termination of competence; growth Resistance to heat, oxidative stress, supobtimal temperature; inhibition of σ^X Colonization of the lung and nasopharynx; virulence in septicemia and pneumonia murine models and intraperitoneal infection; proliferation in lung and blood; intracellular survival in macrophages; virulence factor expression; Protective effect (vaccine development)	[87-88, 106, 120-126]
	ClpC	+	Growth; cell division; autolysis; competence Adhesion to human lung cells; virulence in mouse pneumoniae model; proliferation in lung and blood	[103, 106, 121, 127]
	ClpE	+	Resistance to heat Virulence in intraperitoneal infection; adherence to epithelial cells	[87-88, 106, 128]
	ClpX	-	Essential gene	[106]
	ClpL	+	Growth; autolysis; Heat-shock response Inhibition of adherence and invasion of epithelial cells; survival in macrophages; induction of TNF-α	[87, 106, 129, 130]
S. mutans	ClpP	+	Growth; competence; chain morphology; aggregation; autolysis; inhibition of biofilm in sucrose Resistance and adaptation to heat, acid and oxidative stress; degradation of IrvR	[91, 105, 131-132]
	ClpC	+	Degradation of IrvR	[91, 132]
	ClpE	+	Biofilm formation in sucrose	[91, 105]
	ClpX	-	Growth; aggregation; chain morphology; autolysis; inhibition of biofilm in sucrose resistance to heat and acid stress; degradation of IrvR	[105, 132]
	ClpL	-	Growth; inhibition of biofilm in sucrose Acid tolerance	[105, 133]

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2.5.2. Stress response

As mentioned above, expression of most of the Clp proteins is repressed by the heat-shock response regulator CtsR upon heat stress, leading to CtsR degradation and subsequently enhanced *clp* gene expression. Therefore, Clp proteins are expected to be involved in resistance to heat-shock. So far most of the *clp* mutants described are more sensitive to high temperature exposure with the exception of the *clpC* mutant in *S. pneumoniae* where no striking difference in survival at higher temperature was observed. Therefore, in *S. pneumoniae* the heat-shock response seems to be mediated by the ClpEP complex instead of ClpCP [88, 103, 123]. The other exception is ClpX, which is not a member of the CtsR regulon and the transcriptional expression of which is not increased upon heat-shock. In fact, it was shown in *S. aureus* that the *clpX* mutant was more resistant than the wild-type strain during exposure at 45°C [101]. On the other hand, in *S. aureus* the *clpC*- and *clpP*-deficient strains are sensitive to heat-shock and ClpX and ClpP but not ClpC are required for tolerance to oxidative stress, osmotic stress and suboptimal temperature exposure. This indicates that in *S. aureus*, ClpC and ClpP mediate the response to heat-shock while ClpX is associated with ClpP to mediate the response to oxidative, osmotic and cold stress [83, 101]. The oxidative response is as well mediated by ClpXP in *S. mutans* [91, 105]. In *L. monocytogenes*, resistance to heat is mediated by the ClpCP and ClpEP complexes whereas the ClpB chaperone is involved in tolerance to suboptimal temperature. The ClpCP complex is as well involved in the response to high salt concentration allowing *L. monocytogenes* to grow in processed food [99, 104, 111].

2.5.3. Infection and Virulence

So far, no *clp*-deficient strains have been associated with an increase in virulence in Gram-positive pathogens. Moreover, immunization with ClpP was shown to elicit an immune response that protected mice against pneumococcal infection emphasizing the fact that Clp proteins are important factors in virulence [120, 122]. In *L. monocytogenes*, ClpC is involved in the adhesion and invasion of epithelial cells and in association with ClpP required for the escape of the phagosome and intracellular replication [85]. However in *S. aureus*, intracellular replication requires the ClpXP complex [83]. In addition, a study in *S. aureus* showed that transposon disruption of *clpX* reduces virulence in a bacteraemia mouse model [118]. Comparably, ClpX and ClpP but not ClpC are required for infection in a skin abcess model [101]. This could be explained partly by the decrease in intracellular replication of *clpX*- and

clpP-deficient strains in bovine cells [83]. In *S. pneumoniae*, a large scale mutagenesis in a pneumonia mouse model identified ClpC as required for virulence. Later on, ClpC and ClpE were shown to be involved in the adherence to human lung cells [103, 127]. Comparably, ClpP was shown to be required for virulence in a septicemia and pneumonia mouse model. In fact, the *clpP*-deficient strain was defective to colonize the lung and nasopharynx and to replicate inside the macrophages explaining the reduced virulence [122-123].

The effect of Clp proteins on virulence can be explained in some cases by their role in response to stress widely encountered during infection. For example, the fact that the *S. pneumoniae clpP*-deficient strain failed to survive in the acidic phagosome of macrophages can be a result of the increased sensitivity of the mutant to oxidative stress [121-122]. However, variation of virulence factor and transcriptional regulator expression was observed indicating that Clp proteins are involved in the regulation of virulence. For instance, ClpC is required for the transcription of major virulence factors (*inlA*, *inlB* and *actA*) involved in cell invasion in *L. monocytogenes* confirming its role in adhesion [85]. The same is observed in *S. pneumoniae* where ClpC is required for the transcription of adhesins and pneumolysins [103]. Interestingly, in *L. monocytogenes*, *clpC* is downregulated by the main transcriptional activator of virulence factor PrfA [134]. However, the mechanism by which the Clp proteins interfere with virulence is best characterized in *S. aureus*. Indeed, ClpXP is required for the transcriptional regulation of numerous virulence factors such as haemolysins, extracellular proteases and surface adhesins [101, 114-115]. This regulation is linked to the quorum sensing two-component system Agr, a major and extremely complex regulator of virulence in *S. aureus* [135]. Upon signal sensing, activation of the *agr* operon leads to the production of the effector molecule, RNAIII, a small regulatory RNA responsible for gene regulation. ClpX and ClpP were shown to activate the transcription of RNAIII [114]. Moreover, it was suggested that RNAIII may bind directly to regulatory proteins, thereby inducing conformational changes that destine the protein for degradation by ClpXP. Noteworthy, ClpX independently of ClpP is essential for transcription of *spa*, encoding a cell wall associated protein, Protein A [101, 114]. As Rot is a known activator of *spa* transcription, the authors suggested that ClpX might be required as a chaperone for expression, folding or dimerization of the transcriptional regulator Rot [114, 117, 136].

2.6. Antibiotic therapy

The spread of antibiotic resistance impairs treatment of life threatening diseases. The emergence and increase of multi drug-resistant Gram-positive bacteria such as *S. aureus, S. pneumoniae* and *Enterocccus* causes special concerns and new antibacterial agents are urgently needed. Acyldepsipeptides (ADEPs), isolated from the fermentation broth of *Streptococcus hawaiiensis*, were shown to have antibacterial activity against Gram-positive bacteria including *S. aureus, S. pneumoniae, S. pyogenes* and *Enterococcus faecalis* [137]. Studies showed that ADEPs interact with ClpP abolishing the binding of Clp ATPases, therefore preventing the degradation of folded proteins by ClpP. Thus, cells treated with ADEPs exhibit typical *clpP*-deletion phenotypes such as inhibition of cell division [137-138]. The interaction of ADEPs with ClpP induces conformational changes, which enable ClpP to degrade unfolded proteins without the help of an ATPase. Therefore, ClpP degrades nascent chains exposed at the ribosome leading to the death of the bacteria [138]. Consequently, ADEPs are the first naturally occurring molecules activator of a proteolytic machinery suggesting a new mechanism by which an antibiotic can trigger bacterial death. In contrast to other antibiotics that inhibit essential cellular functions, ADEPs trigger bactericidal activity by turning a tightly regulated peptidase into an uncontrolled protease.

3. Streptococcus pyogenes

S. pyogenes also known as group A streptococcus (GAS) is a human Gram-positive extracellular pathogen. GAS is a non-motile, non-spore-forming bacterium that appears as pairs or moderate-sized chains (2-30 cells in length). The streptococcal cell is spherical or ovoid and has a diameter of 0.6-1.0 μm. Colonies can be easily distinguished when cultured on blood agar plates, appearing white, translucent, sometimes mucoid surrounded by a hemolytic zone. It is a facultative anaerobic bacterium with a fermentative metabolism, auxotrophe for most of the amino acids. The Lancefield classification identified *S. pyogenes* as group A streptococcus based on its surface carbohydrate (*N*-acetyl-β-D-glucosamine link to a polymeric rhamnose backbone). Group A streptococci were then classified based on their surface M protein [139]. The M protein typing, based on the different alleles of *emm,* encoding M protein, is the most diverse up to now, with more than 150 M serological types identified. The T-typing, based on the diversity of *tee,* the T protein antigen encoding genes, can be used in addition to M protein, to classify strains [140]. Alternatively, GAS is also

subclassified with regard to the expression of the serum opacity factor (OF), an enzyme that causes mammalian serum to increase in opacity.

3.1. Diseases and persistence

S. pyogenes can colonize the throat and skin and cause a wide variety of human diseases. Infections due to GAS range from common clinical illnesses including pharyngitis, tonsillitis, cellulitis, impetigo and scarlet fever to severe life threatening invasive infections such as sepsis, necrotising fasciitis and streptococcal toxic shock syndrome. Moreover, GAS is associated with the development of post-streptococcal sequelae including acute rheumatic fever or acute glomerulonephretis (Table 4) [141]. These sequelae are explained by the fact that several streptococcal virulence factors are mimicking human molecules therefore inducing an autoimmune response [142]. Interestingly, M serotypes causing skin infection are in general different from those causing pharyngitis [143].

The late 1980's saw the resurgence in the USA and Europe of severe diseases involving *S. pyogenes*, despite the sensitivity of the pathogen to penicillin. The reemergence of toxic shock syndrome and "flesh eating" disease, fast progressing invasive diseases with high mortality rates ranging from 30% to 80%, could be linked to new or reemerging serotypes. Several different M types were isolated with a domination of M protein serotype 1. Acquisition of new genetic material through horizontal gene transfer of bacteriophages has led to new and more aggressive streptococcal strains. Indeed, bacteriophages count for 7.1% of the M1 serotype genome and carry genes coding for important virulence factors including superantigens SpeA and SpeC [144]. New combinations of genes or allelic variants results in genetic diversity that might increase the fitness of the bacterium by conferring unique properties [8, 145]. Interestingly, studies showed that the same strain can induce different symptoms in infected individuals [146-147]. This could be explained by variations of the host immunogenic factors conferring predisposition or protection against streptococcal infections [148]. Penicillin is still the drug of choice to beat GAS infections. So far no resistance to penicillin has developed in GAS strains. However, some strains showing tolerance to penicillin arised, although the relative infections can be treated with higher amounts of the antibiotic. The production of a safe and effective vaccine is still in progress. The most promising approaches are targeting the conserved region of the M protein to avoid serotype specificity [149-151]. Altogether, GAS causes immense human morbidity and mortality and remains a significant public health problem.

Superficial skin and soft- tissue infections
Cellulitis Pharyngitis Eryipelas
Invasive Group A streptococcal diseases
Streptococcal toxic shock syndrome (STSS) Necrotizing fasciitis (NF) Bacteremia Osteomyelitis Septic arthritis Pneumonia
Complications of GAS illness
nonsuppurative Acute Rheumatic fever Poststreptococcal glomerulonephritis **suppurative** Cervical lymphadenitis Endocarditis Fasciitis/myositis syndrome Mastoiditis Meningitis Otitis media

Table 4. Diseases caused by GAS. Adapted from [141].

3.2. Infection cycle

S. pyogenes is considered as an extracellular human pathogen. The most important step for the onset of infection is the attachment of GAS to throat or skin epithelial cells. Therefore, the bacterial cell must compete with the existing tissue flora and avoid clearance through mucous and salivary fluids. The adhesion is a combination of a first weak interaction with the mucosa mediated by the lipotheichoic acid followed by a strong tissue specific adherence involving multiple adhesins that includes M protein, fibronectin binding protein and hyaluronic acid capsule [152]. After adherence, GAS must maintain itself at the site of infection. Thus, *S. pyogenes* can form aggregates directly on the tissue promoting microcolonies and biofilm formation [153-154]. Strains with the ability to form these aggregates persist longer in the host [155]. For long thought to be an extracellular pathogen, *S. pyogenes* was shown to be able to invade epithelial cells at frequencies equal or greater than *L. monocytogenes*, a classical intracellular pathogen [156-157]. Internalization enables GAS to avoid host immune response and antibiotic mediated killing and persist in the organism [158]. It is possible that penetration into epithelial cells is the first step to deeper

tissue invasion. The next step of infection is to escape the host immune response by developing mechanisms against opsonization and phagocytosis. The antiphagocytosis behavior of *S. pyogenes* involves the inhibition of the complement activation via the binding of M protein to the factor H or the cleavage of the complement component C5a. Moreover, GAS exhibits at its surface proteases able to cleave specifically immunoglobulins. Finally, severe streptococcal diseases are linked to the spreading of GAS in the blood stream and/or through tissues. To achieve this goal, GAS secretes a variety of molecules involved in the dissolution of clots, degradation of DNA or disruption of cellular junctions leading to complete tissue destruction.

3.3. Virulence factors

Virulence factors are defined as factors allowing an organism to set in the host and maintain the bacteria after initial infection. These factors support adherence at the site of infection, allow internalization into epithelial cells, enable the bacteria to escape from the immune response and promote tissue damage. The main streptococcal virulence factors are listed below and grouped in two categories: factors associated to the cell wall and those that are released from the cell (Table 5).

3.3.1. Cell wall-associated virulence factors

3.3.1.1. M protein

M protein is the best characterized streptococcal virulence factor. It is composed of two polypeptide chains complexed in an alpha-helical coiled-coil configuration anchored in the cell membrane, traversing the cell wall and appearing as fibrils on the cell surface. The C-terminal end of the protein located in the cell wall is highly conserved among *S. pyogenes* strains whereas its hypervariable amino terminal end extends in the environment. The hypervariability of the N-terminal region constitutes the base of the M protein typing system [139].

The main role of M protein is the inhibition of phagocytosis. Streptococcal strains lacking the *emm* gene are phagocytocized more efficiently [159]. M protein binds the factor H, thus impeding the binding of the C3b to the bacterial surface therefore inactivating the complement response and the recognition by polymorphonuclear phagocytes, PMNs [160-161]. Futhermore, M protein acts as an adhesin by binding fibronectin and mediates adherence to keratinocytes via attachment to the CD46 located on their membrane [161-162].

M protein is mainly expressed during the first stage of infection. Its expression is regulated by the major regulator Mga whose encoding gene is located upstream of *emm*, among other regulators [163-164].

Adherence	Internalisation
Lipoteichoic acid (LTA)	M protein
Fibronectin binding proteins	Protein F1
M protein	**Invasion**
Hyaluronic acid capsule	Hyaluronic acid capsule
Collagen binding protein	M protein
Pili	**Spread through tissues**
Antiphagocytocic	Streptokinase
M protein	Cysteine protease SpeB
M-like protein	DNase SdaI
Hyaluronic acid capsule	Hyaluronidase
Immunoglobulin degrading enzyme IdeS	**Systemic toxicity**
C5a peptidase	Streptolysin O (SLO)
Streptococal inhibitor of complement (Sic)	Streptolysin S (SLS)

Table 5. Streptococcal virulence factors. Adapted from [165].

3.3.1.2. M-like proteins

The genes *enn, mrp, fcrA, arp* and *prtH* are among the members of the *emm*-like gene family. It has been proposed that a common ancestral gene underwent duplications leading to the diversified family of M-like proteins [166-168]. They code for proteins, which are structurally similar to M protein especially in the C-terminal region [169]. They bind a wide range of host proteins such as albumin, fibrinogen, immunoglobulin and plasminogen participating along with M protein to the antiphagocytic response and adhesion of GAS to the specific tissue at the site of infection [142, 170-171]. As for *emm*, expression of the *emm*-like genes is regulated by Mga [172].

3.3.1.3. C5a peptidase

The C5a peptidase or ScpA is an endopeptidase that specifically cleaves the complement component C5a, thereby inhibiting the recruitment of PMNs and protecting the organism from immune detection [173]. ScpA mutant strains exhibit a decrease in colonization of the mouse nasopharynx [174]. The size of the *scpA* gene varies among the

different GAS serotypes due to a variation of the number of repeat units [175]. ScpA is mainly expressed during exponential phase and its expression is activated by Mga.

3.3.1.4. Capsule

The hyaluronic acid capsule promotes resistance against phagocytosis [176]. Strains lacking the capsule exhibit a reduced virulence in various animal models. Moreover, the hyaluronic capsule binds CD44 on epithelial cells indicating a role in adherence [177]. The binding to CD44 can induce cytoskeleton changes resulting in disruption of intercellular junctions thereby promoting the invasion of the epithelium [178].

The amount of capsule varies between the different streptococcal strains, and more virulent strains produce higher amounts of capsule displaying a mucoid appearance when cultivated on agar plates [179]. The capsule is highly produced during the early-exponential phase. The streptococcal capsule is composed of a polymer of hyaluronic acid containing units of glucoronic acid and *N*-acetylglucosamine [180]. The three genes involved in the synthesis of the polymer are grouped together in the same locus and expressed as an operon [181]. The *has* operon is composed of *hasA* encoding a hyaluronate synthase, *hasB* coding for a UDP-glucose deshydrogenase and *hasC* encoding a UDP-glucose pyrophosphorylase. Interestingly, *hasA* and *hasB* are sufficient for a standard capsule expression indicating that an alternative source of UDP-glucose could be used for capsule production while *hasC* is not necessary for hyaluronic acid synthesis [182]. The operon is expressed as a single transcript from the promoter located upstream of *hasA* and is negatively regulated by the two component system CovR/CovS [183-186].

3.3.1.5. Adhesins

S. pyogenes expresses at least 17 different kinds of adhesins [187]. The expression of these adhesins can differ between strains, the site of infection or environmental factors.

The lipoteichoic acid (LTA) binds to fibronectin using hydrophobic interactions. It has been postulated that LTA brings the organism into close contact with the host cell and then other adhesins promote higher affinity binding [152, 188].

The protein F1 also called SfbI binds fibronectin and is involved in the adherence to respiratory epithelial cells [189]. Moreover it plays a major role in the adherence to cutaneous Langerhans cells [162]. SfbI expression is enhanced by oxygen indicating that SfbI is displayed in case of cutaneous adhesion.

In addition, other fibronectin binding proteins have been identified to act as adhesins including, SfbII, FBP54, PFBP and protein F2 [190-193].

3.3.1.6. HtrA

HtrA is a heat-shock induced envelope associated serine protease. Its *E. coli* homologue, DegP, located in the periplasm, is responsible for the degradation of abnormal periplasmic proteins [194-195]. In the absence of periplasm in Gram-positive bacteria, HtrA is associated with the membrane. The protease is involved in the regulation of the secreted virulence factor SpeB by activating its maturation and in the control of hemolysin S by inhibiting its activity during the exponential phase [196].

3.3.1.7. Pili

For long, *S. pyogenes* was thought to be non piliated. However, in 2005 a study highlighted the presence of pilus-like structures at the surface of the bacteria [197]. The GAS pili are encoded by a highly variable 11 kb pathogenicity island known as the fibronectin-binding, collagen-binding, T-antigen region (FCT). This region contains genes coding for the pili backbone protein, ancillary proteins and sortase. The backbone protein harbors a LPXTG motif, commonly found in adhesins [197]. Unsurprisingly, streptococcal pili are involved in adhesion to epithelial cells and biofilm formation [154].

3.3.2. Secreted virulence factors

3.3.2.1. Streptokinase

A common defense mechanism employed to prevent systemic dissemination of invasive bacteria involves occlusion and encapsulation of bacteria within fibrin networks. Streptokinase or Ska converts plasminogen into its active form plasmin therefore promoting the dissolution of clots that facilitate the spread of the bacteria in the host [198]. To improve the spreading of the bacteria and the bacterial toxins, the streptokinase-plasmin complex degrades fibrin clots as well as extracellular matrix. Streptokinase is highly specific for human plasminogen, exhibiting little or no activity against other mammalian species, including mouse [199].

3.3.2.2. DNases

The Streptococcal DNases were one of the first secreted proteins to be identified [200]. However, their role in virulence remained vague for decades. Every serotype produces

at least one DNase and most strains produce several distinct enzymes [201-202]. Their expression is upregulated after interaction with epithelial cells and PMNs [203-204]. The discovery of secreted neutrophil extracellular traps (NETs), composed of chromatin and granulocyte components, which capture and kill bacteria, shed some light on the role of DNase in GAS virulence [205]. Indeed, it was shown that the bacteriophage located *sdaI* gene, encoding the main DNase in the GAS M1 serotype, was necessary to escape NETs therefore limiting the PMN-mediated killing [206-207].

3.3.2.3. Streptococcal inhibitor of complement

Along with M protein and C5a peptidase, the streptococcal inhibitor of complement (Sic) interferes with the complement response. Sic binds the membrane insertion site on complement C5b-C7 complex therefore inhibiting the complement mediated lysis [208]. In addition, Sic inactivates two main antibacterial peptides involved in the innate immune response, LL37 and alpha-defensin [209]. Two other components of the innate immune response, proteinase inhibitor and lysozyme can intereact with Sic leading to the inhibition of their activity [210]. In addition, Sic enhances bacterial survival by binding the eukaryotic plasma membrane protein Ezrin. This interaction alters the internalization and PMN-mediated killing of *S. pyogenes* [211]. *sic* is located in the vicinity of the transcriptional regulator *mga* and was identified to be part of the Mga regulon [146].

3.3.2.4. Cysteine protease SpeB

The streptococcal cystein protease or pyrogenic toxin B (SpeB) is one of the best characterized streptococcal virulence factors. The fact that SpeB is present in all GAS strains and highly conserved, suggests its importance for the virulence of *S. pyogenes* [212-213]. As a result of its protease activity, SpeB is involved in the cleavage of proteins such as immunoglobulin, vitronectin, fibronectin and the complement component C3 [214-215] and induces active peptides such as interleukin-1, kinins and histamine [216-217]. Futhermore, SpeB cleaves and thereby inactivates the antimicrobial peptide dermatan sulphate on the extracellular matrix [218]. In parallel, SpeB cleaves streptococcal surface proteins either to release them from the surface promoting their dissemination in the host (M protein, C5a peptidase, protein H) or in the case of Ska, SdaI, Protein F1, to degrade them when not needed anymore [219-222].

SpeB is produced as a 40 kDa zymogen and then processed to a 28 kDa mature peptide [77]. The maturation of SpeB is complex, involving a combination of *cis*- and *trans*-

processing from SpeB itself leading to eight intermediates. SpeB is also regulated by other streptococcal proteins such as HtrA, a surface anchored streptococcal serine protease necessary for the maturation of SpeB, however its role remains unknown [196, 223]. In addition, M protein was shown to be necessary for the proper folding of SpeB [224].

3.3.2.5. IdeS

IdeS or Mac is another cysteine protease, which cleaves specifically the heavy chain of immunoglobulin IgG [225-226]. The other classes of human immunoglobulins IgA, IgD, IgE and IgM are not affected by IdeS. To date no other substrate of IdeS has been identified. IdeS is mainly expressed during exponential phase being a better candidate than SpeB for cleaving opsonizing IgG as soon as they reach the GAS surface [225, 227].

3.3.2.6. Streptolysins

GAS secretes two streptolysins and encodes three other putative ones based on homology to hemolysins of other organisms [144].

The streptolysin O (SLO) is an oxygen sensitive, thiol activated toxin produced by nearly all GAS isolates. SLO is binding and aggregating onto the cholesterol of the host cell membranes leading to the formation of pores [228]. Target cells include among others, erythrocytes, macrophages and leukocytes.

Produced by most of the GAS strains, the streptolysin S (SLS) is an oxygen stable non-immunogenic toxin that is released from the cell upon presence of the appropriate carrier molecule. The mechanism of SLS mediated lysis is similar to the one of the complement: a lysine complex introduced into the membrane leads to the formation of pores [229-230]. SLS is encoded by a nine gene operon where the first gene *sagA*, codes for a 53 amino acid peptide responsible for the hemolytic activity [231-232]. The downstream genes, *sagB* to *sagI* are required for the proper processing and export of SLS. The pleiotropic effect locus (*pel*) is located at the *sag* operon. *pel* includes the *sagA* gene and codes for a regulatory RNA involved in the regulation of the *sag* operon as well as *speB, emm* and *sic* [233].

3.3.2.7. Streptococcal superantigens

The streptococcal superantigens or streptococcal pyrogenic exotoxins are believed to be associated with the streptococcal toxic shock syndrome. The family of exotoxins includes the bacteriophage-encoded SpeA and SpeC, SpeG, SpeH, SpeJ, SpeK, SpeL, SpeM, SSA, SMEZ and SMEZ-2. The crystal structures of the superantigen are highly similar to each

others and to staphylococcal superantigens [234]. Exotoxins are able to simultaneously bind to the MHC class II molecules and the T-cell receptor leading to the secretion of excessive amounts of inflammatory cytokines responsible for the toxic shock syndrome.

3.4. Regulators

Unlike other Gram-positive bacteria such as *S. aureus* or *L. monocytogenes*, *S. pyogenes* does not appear to use alternative sigma factors to regulate virulence gene expression in response to stress or growth [235]. It depends instead on various transcriptional regulators responsive to environmental changes.

3.4.1. Stand-alone regulators

The term "stand-alone" regulator was first used in 2003 by Kreikemeyer and colleagues to describe transcriptional regulators controlling a multigene regulon in response to environmental changes [236]. Mga, RAPLs and RopB were identified as stand-alone regulators. GAS exhibits a growth phase-dependent profile of virulence factor expression controlled mainly by these regulators. The Mga regulon is mainly expressed during exponential phase thus likely to promote colonization of the host [237]. When encountering stationary phase, RAPL and then RopB inhibit the Mga regulon, thus likely to promote the transition from colonization to persistence and spread in the organism [238-239].

3.4.1.1. Mga

The multigene regulator Mga, previously called VirR, is encoded upstream of *emm* and was identified as the positive regulator of M protein expression [163, 240]. Mga is ubiquitous in GAS and has been found in all strains examined up to now [241]. Mga is growth phase dependently regulated. Its expression is maximal during exponential phase and is environmentally regulated by aeration conditions [237]. Indeed, carbon dioxide is stimulating the expression of *mga* [242]. Furthermore, Mga is necessary during the first stage of infection enabling colonization and escape from the immune response by activating the expression of genes involved in adherence, internalization into non phagocytic cells and evasion of the host immune response.

Various studies show that Mga positively regulates the expression of several virulence factors mainly surface-associated factors such as C5a-peptidase, serum opacity factor, glycoprotein binding protein, fibronectin binding protein (Fba, Sfb), collagen binding protein (SclA) and the secreted inhibitor of complement (Sic) [172, 243-247]. In addition to virulence

factors, Mga positively regulates the *opp* and *dpp* operons, involved in nutrition by transporting small peptides into the cell [248-249]. Interestingly, *opp* and *dpp* operons upregulate the expression of *speB*, establishing that Mga is indirectly involved in the regulation of the cysteine protease SpeB. Moreover, microarray analysis of the Mga regulon showed that Mga additionally regulates operons involved in the transport and utilization of carbohydrates and iron [247]. Thus, Mga has a major impact not only in the regulation of virulence but also in metabolism.

Mga directly binds DNA in the promoter region of the genes it regulates. Mapping of the binding sites of Mga in the promoter region of *emm*, *emm-like* genes and *scpA* led to the identification of a 45 bp consensus Mga-binding site overlapping the -35 region and allowing the interaction with the α-subunit of the RNA polymerase [250-251]. Mga also regulates its own transcription via binding to its promoter via a distinct activation mechanism [164]. *mga* is expressed via two promoters a distal promoter P1 and a proximal promoter P2. The binding of *mga* is required for the activation of transcription via the major promoter P2 as well as for the inhibition of expression via the distal promotor P1. The expression via the distal promoter is activated early in growth by CcpA, the catabolite repression regulator initiating the Mga response [252]. On the other hand, RopB and RofA, two transcriptional regulators were identified as negative regulators of *mga* expression [238-239]. Ribardo and colleagues showed that AmrA, a membrane protein involved in cell wall synthesis, is required for the maximal expression of the Mga regulon during exponential phase, thus linking *mga* expression to the cell wall turn-over [253].

3.4.1.2. RAPLs

RofA-like proteins (RAPL) are a family of transcriptional factors that possess a high homology to each other. The genome of *S. pyogenes* counts 4 RAPLs: RofA, Nra, RALP-3 and RivR.

- RofA

RofA for regulator of the fibronectin binding protein (Protein F) was identified in a strain where Protein F was constitutively expressed [254]. Further studies showed that RofA is involved in the positive regulation of protein F1 but is negatively regulating the expression of *speA*, *sagA* and the transcriptional regulator *mga* [238, 255]. A *rofA* knock-out strain exhibits reduced attachment and internalization to epithelial cells and decreased host cell viability. The gene coding for the protein F, *prtF* and *rofA* are adjacent on the chromosome

but divergently oriented. RofA is autoregulated and binds to a consensus sequence of 17 bp (TTTTCACCAAAAANCAT) present in the promoter of its gene as well as the one of *prtF* [256]. This sequence was also identified in the promoter of the pilus associated protein coding gene *cpa*. However, the RofA binding site is absent in the promoter region of *speA, sagA* or *mga* indicating that the effect of RofA on these genes might be indirect. Moreover, whereas *mga* is activated by carbone dioxide, RofA responds to increased oxygen level [257].

- Nra

Nra, a negative regulator in GAS, possesses 62% sequence homology to RofA. Unlike RofA, Nra expression is not affected by the level of oxygen. Nra negatively regulates the expression of the fibronectin binding protein F2, the collagen binding protein Cpa, SpeB, SpeA, SLS, Mga and its own expression [258-259]. Transcriptome and proteome analysis increased the Nra regulon by adding genes coding for pilus biogenesis and capsule synthesis, along with other stand alone regulators such as RopB and other RAPL members, RAPL3 and RivR [260]. Nra inactivated strains exhibit an increase in adherence and internalization and escape more efficiently the phagosome [259].

- RAPL3

RAPL3 has been identified in some specific serotypes, such as M1 and M49. RAPL3 is located upstream and divergently transcribed from *lsp* encoding a fibrinogen binding protein and in the vicinity of *sagA* [261]. RAPL3 represses the expression of *lsp*, the *has* operon and *speB*.

- RivR

Finally, RivR, the last RAPL was shown to activate the expression of *mga* and the Mga regulon [262]. Interestingly, RivR is directly repressed by CovR/CovS linking the two component system and the Mga regulon. RivR is associated with a small RNA, *rivX*, involved in the regulation of *mga* [263].

Despite the high homology within the RAPL family, each member contributes to the regulation of bacteria-host attachment, internalization and intracellular persistence throughout different stages of bacterial growth.

3.4.1.3. RopB

Members of the Rgg family of transcriptional regulators can be found in many Gram-positive bacteria including *S. gordonii, S. oralis, Lactococcus lactis* or *S. pyogenes* [264-266].

A common feature of these orthologues is the transcriptional regulation of neighboring genes by binding to their promoter region [267-268].

The Rgg orthologue in GAS, *ropB,* is located adjacent to and divergently transcribed from *speB* [269]. Nevertheless, RopB positively regulates the expression of SpeB. Indeed, in the absence of *ropB,* the expression of *speB* is completely abolished [265]. Several transcriptome and proteome studies clarified the RopB regulon [239, 270-273]. In addition to *speB,* RopB activates the transcription of *grab*, the superantigen *speG*, autolysin and lysozyme. On the other hand, RopB represses the expression of SLO, SLS, Nga, secreted DNases MF, HtrA protease and the Mga regulon (*sic, sclA, scpA, emm, ska*). However, RopB does not only interfere with virulence factor expression, it is also involved in the control of streptococcal regulators. As previously mentioned, RopB inhibits the expression of Mga leading to complete blockage of its regulon expression at stationary phase and activates the expression of several two-component systems, including CovRS, FasBCA and Irk/Irr.

RopB is additionally involved in the repression of amino acid catabolism operons including arginine, histidine and serine [271-272], while it is essential for the growth in non glucose sugar conditions [274]. During infection, the concentration of glucose in the host (i.e. nasopharynx) is too low for GAS to survive, which results in the use of an alternative carbon source mediated by RopB, highlighting its major impact for survival. Thus, RopB is essential for the proliferation of GAS in the host. In addition, RopB interferes in the heat-shock and oxidative response by repressing the expression of several genes important for thermal (*clpE, clpL*) and oxidative stress (*ahpCF*) [272]. Another study shows that *ropB* deficient strains are more resistant to penicillin, thus emphasizing the role of RopB in response to antibiotic treatment [275].

RopB exhibits a helix turn helix domain and was shown to bind to the intergenic region of *speB* and *ropB* leading to the transcriptional regulation of both genes. This indicates that *speB* and *ropB* share the same promoter region [269]. So far, no other binding site has been identified and no consensus binding sequence has been proposed.

Table 6: *S. pyogenes* regulators. UP: up-regulated; DW: down-regulated. Updated from M. Siller's Ph.D thesis manuscript 2008

Stand-alone regulators		
CodY	UP: pel/sagA, nga-slo, mga, grab, scl, prtS, scpA, speH, ideS, hasA DW: covRS, ropB, opp, ska, sda	[280-281]
LacD.1	UP: manL, slo, sagH, ntpK DW: speB, salA, scrA	[282]
MalR	DW: transcripts of polysaccharide utilization proteins.	[283]
Mga	UP: arp, emm, enn, scpA, fcrA, nra, mga, ska, speB, scnA/salA, opp, fbA, sic, lbp, scl1/scl-A, sof, mrp DW: genes for sugar utilization	[164, 236, 284-285]
MsmR	UP: prtF2, cpa, nra, spy0128, nga, spy0166, slo, spy0170, spy2006, sof, sfbx, hasA	[286-287]
MtsR	UP: htsA, mga, ska, emm, SpeB DW: sia operon, mtsA, PrsA	[288-291]
RALP3	DW: hasA, spn, sdal, lsap, speB, mga, slo, covR, sic, sagA, grab, emm, eno (at logarithmic phase), ska, scpC UP: scpA, cpa, eno (at stationary phase), sic	[260-261]
RALP4 (RivR)	UP: mga, emm, scpA, fba, sic, scl, grm speB, spy1508/NT01sp1245,5005_spy0190/NT01sp0244 and NT01sp1815 (hypot. proteins)	[263]
Nra	DW: cpa, mga, nra, prtF2, operon orf5-nifR3L-kinL, operon cpa-lepAL-egfflSL-orf2 (pilus gene), sof/sfbII, sagA, speA, speB, ralp3	[236, 258-260]
RofA	UP: prtF, rofA, rpsL, hasB, emm2 DW: emm6, mga, sagA, speA, speB, ska	[236, 238, 254]
Rgg/RopB	UP: speB, autolysin, clpB, lysozyme, covR, covS, fasBCA, isp1, isp2, ihk, irr DW: mf, DNA entry nuclease (orf226), orf953, emm, grab, hasAB, orfX, sagA, scl1, scpA, ska, slo, speH, mac, mga, cpsX, yufM, lytR, spy0875, ClpE, ClpL, HtrA, AphCF	[239, 265, 269, 272-273, 292-293]
PerR	UP: csp, sod, czcD, Dpr DW: mtsA, mrgA, pmtA, phtY, phtD, lsp, lrpsN2	[294-296]
P-Ser-HPr	UP: mga	[284]
Srv	UP: sir, spy0044 (zinc containing dehyDWogenase), spy0285 (ATP binding protein), spy0714 (zinc binding protein), spy2007, sic, speB	[278-279]
Two-component systems		
CovRS	UP: spy0138, spy1062, spy1680, spy1755, spy533 DW: covR, grab, has-operon, dppA, ideS, ihk/irr, isp2, lmb, mac, mspA, ralp3, sagA, sda, ska, speB, speF, speMF, trxSR, rivR	[184-185, 262, 297-301]
fasBCAX	UP: ska, SLS activity DW: fbp45, mrp, sagA	[302]
Ihk/Irr	UP: cytokine genes, fbp, gidB, mf/mf3, mryY, sagA, spy0510, spy1035, spy1093, spy1205, spy1311	[204, 303]
SilA/B	UP: silE/D/CR, spyM3-1016 (transposase), bacteriocin like peptide (blp) DW: silC	[304-305]
SptRS	UP: carbyhyDWate metabolism enzymes, emm, hasA, perR, rofA, sagA, sic, spd, speB, spy0470	[306]
VicRK	UP: putative cell wall hyDWolase gene pcsB, putative phosphotransferase system enzyme II for carbohyDWate transport. DW: spy0183, spy0184 (osmoprotectant transporter OpuA	[307]
Regulatory RNAs		
fasX	UP: ska, SLS activity DW: fbp45, mrp, sagA	[302]
pel	UP: emm, nga, sic, SpeB activity	[233]
rivX	UP: mga, emm, scpA, fba, sic, scl, grm (Mga regulated genes), speB, spy1508/NT01sp1245, M5005_spy0190/NT01sp0244 and NT01sp1815 (hypot. proteins)	[263]

3.4.1.1. Srv

The streptococcal regulator of virulence (Srv) is a member of the Crp/Nfr family of transcriptional regulators and share 53% sequence homology with the *L. monocytogenes* positive regulator factor A (PrfA) [276]. Srv is involved in biofilm formation and therefore inactivation of *srv* results in a decrease in biofilm [277]. Moreover, it was shown in mice

infection models that Srv is necessary for the full virulence of GAS [278]. Microarray analysis revealed that Srv slightly represses virulence gene expression but its impact on their transcription is really modest [279].

3.4.1.2. CodY

CodY is a pleiotropic transcriptional regulator involved in the response to nutrient limitation in a RelA-independent pathway [281]. It is highly conserved in low-G+C Gram-positive bacteria and activated by branched-chain amino acids [308]. As response regulator for nutrient limitation, CodY directly or indirectly controls the expression of many genes coding for transporters and metabolic enzymes as well as virulence factors. In addition, CodY influences the expression of regulators of virulence factors including *ropB, mga, covR* and *pel* and their regulon [280-281].

3.4.2. Two-component systems

A two-component system is based on two proteins: a sensor kinase that detects the specific signal and phosphorylates a DNA binding regulator, which in turns directly influences target gene expression. Bioinformatic studies revealed the presence of 13 two-component systems encoded in the genome of various strains of *S. pyogenes*. So far not all of them have been functionally characterized. This part will focus on the well studied ones.

3.4.2.1. CovR/CovS

CovR/CovS (control of virulence genes) previously known as CsrR/CsrS (capsule synthesis regulator) regulates more than 15% of streptococcal chromosomal genes [186]. First, CovR/CovS was found to negatively regulate the expression of the streptococcal capsule [184]. Inactivation of *covR,* encoding the repressor molecule, results in an increase of hyaluronic acid capsule production associated with elevated virulence in animal models. Further studies showed that CovR downregulates the expression of several other virulence factors such as *sagA, speMF, speB* and *ska* [185, 299] and also represses its own transcription [185]. Upon ligand binding, CovR is phosphorylated by CovS resulting in the formation of a complex composed of multimers of CovR [309]. This complex binds to a common 16 bp motif [5'-T(T/A)ATTTTTAA(A/T)AAAA(C/A)-3'] conserved in the promoter region of *hasA, speMF, ska, sagA* and *speB* [310-311]. In *S. pyogenes*, CovR/CovS responds to environmental Mg^{2+} but not to Ca^{2+}, Mn^{2+} and Zn^{2+} abundance [312]. Growth in the presence

of Mg^{2+} leads to the repression of capsule production, streptokinase and streptolysin S expression indicating that Mg^{2+} activates CovR via phosphorylation [312].

3.4.2.2. FasBCAX

The FasBCA operon was identified by its homology to the well described *S. aureus agr* and *S. pneumoniae com* operons. The operon codes for 2 potential histidine kinases, FasB and FasC and one response regulator, FasA. The significance of these two histidine kinases remains unclear. Either they sense different signals or they act as a heterodimer. *fasX*, located downstream of the operon, codes for a small RNA regulating the operon.

The FasBCA system regulates fibrinogen/fibronectin binding, hemolytic activity and streptokinase transcription. Inactivation of the *fasBCA* operon results in an increased expression of the fibrinogen binding proteins Fbp54 and Mrp, a decrease activity of Ska and SLS and a reduced transcription of *sod*, coding for the superoxide dismutase. Nevertheless, no changes in expression of *speB, emm, hasB, slo* or *mga* were observed in the *fasBCA* deficient strains [302].

3.4.2.3. Ihk/Irr

The Ihk/Irr two-component system encoding genes are located upstream of *isp*, coding for the immunogenic secreted protein, thus explaning their name: *isp*-adjacent histidine kinase (Ihk) and *isp*-adjacent response regulator (Irr). Ihk/Irr regulates the expression of genes involved in cell wall formation and peptidoglycan synthesis [204], and thus plays a major role in the evasion of GAS from PMN-mediated killing [303].

3.4.3. Regulatory RNAs

In prokaryotes, regulatory RNAs act as signal transducers of environmental conditions to coordinate gene expression. These RNAs carry out a variety of biological functions such as plasmid replication, transposition, viral replication, bacterial replication, and some of them act as regulators of virulence gene expression [313]. In GAS, three loci have been reported to be involved in RNA-mediated control of gene expression. One, termed *fasX* RNA was described to be the effector molecule of the *fas* operon [314]. *rivX*, encoded within the *riv* operon, activates the expression of the Mga regulon [315]. Finally, *pel* located within the GAS *sag* operon (encoding SLS) was identified to exert a pleiotropic effect on the expression of a number of virulence factors [233].

3.5. Proteases in *S. pyogenes*

3.5.1. Proteases

Proteolysis plays a major role in biological processes. It enables inactivation of proteins by degradation but also activation following cleavage of immature forms of proteins. Proteases in pathogenic bacteria participate in many functions essential to virulence, including the acquisition of nutrients, biogenesis of virulence factors, cleavage of key host proteins for modulation of host response, and activation of streptococcal proteins. Several proteases act as virulence factors (i.e. SpeB, IdeS and C5a peptidase).

In addition to the production of several proteases, *S. pyogenes* can recruit human proteases to its surface. The main example is the plasminogen/plasmin, involved in the fibrinolytic system. Plasminogen is recruited to the surface via interaction with cell wall attached proteins and converted to plasmin by the streptococcal secreted streptokinase [316]. The acquisition of plasmin by GAS is important to cross biological barriers such as the extracellular matrix and disseminate in the host.

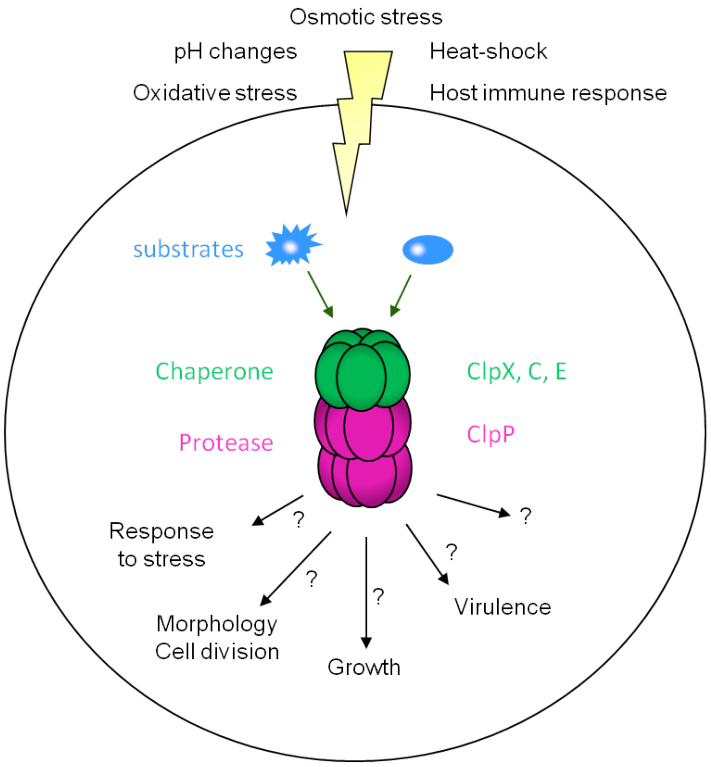
Proteolysis is tightly regulated and depends on the stages of GAS infection. During adhesion, corresponding to the exponential phase *in vitro*, GAS exhibits on its surface proteins that are mainly involved in adhesion and antiphagocytic mechanisms. The protease C5a peptidase is necessary at this stage to escape the complement response. IdeS is mainly expressed during this phase to degrade IgGs as soon as they reach the GAS surface [225]. Later, when GAS colonizes the site of infection, SpeB is produced. It cleaves C5a peptidase, which is not required anymore and adhesins (M protein and fibronectin-binding protein), allowing GAS to leave the site of infection and spread [219].

3.5.2. Clp proteins in *S. pyogenes*

GAS possesses orthologues of the main *clp* genes. Genes coding for the protease ClpP and the chaperone ATPases ClpX, ClpC, ClpE, ClpB and ClpL are present in the genome. Surprisingly, little is known about Clp proteins in *S. pyogenes*. A study identified ClpP as a repressor of σ^x, a secondary sigma factor involved in the regulation of competence [317-318]. Moreover, a microarray study showed that expression of *clpC* and *clpE* is up-regulated under starvation conditions [319]. Finally, as mentioned above, RopB represses the transcription of *clpE* and *clpL* [272].

Aims of the study

The aim of our study is to determine the functions of Clp-mediated proteolysis in the human pathogen *S. pyogenes*. Proteolysis is required in the cell for the natural turn-over of cytosolic proteins as well as in the regulation of metabolic pathways. Various studies have also shown that Clp-mediated proteolysis plays an important role in the regulation of virulence in Gram-positive pathogens such as *L. monocytogenes, S. aureus, S. pneumoniae* and *S. mutans*. However, the impact of Clp proteolysis in the human pathogen *S. pyogenes*, agent of pharyngitis and life threatening diseases such as flesh eating disease and toxic shock syndrome, has not been studied so far. Using in-frame deletion mutants of the Clp ATPases encoding genes *clpC, clpE* and *clpX* and of the protease encoding gene *clpP*, we performed various phenotypic analyses to examine the impact of Clp-mediated proteolysis on growth, cell division and survival to stress conditions encountered during infection. In parallel, we investigated the function of Clp proteins in the pathogenicity of *S. pyogenes* by investigating the expression of virulence factors. Additionaly, we analysed the expression of transcriptional regulators and two-components systems in order to obtain an insight into the mechanism by which Clp proteins influence adaptation and virulence.



Materials and methods

1. Bacterial strains and growth conditions

E. coli strains DH5α was used for the cloning of all constructs prior to their introduction into *S. pyogenes* SF370 (EC904). *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C with shaking (160 rpm). For solid LB medium, 1.5% of agar was added. To select for antibiotic resistance, 25 µg/ml of kanamycin was added to the medium as required.

S. pyogenes strains were routinely cultured in Todd Hewitt Broth (THB, BD) complemented with 0.2% of yeast extract (THY) at 37°C, 5% CO_2 without agitation or on Trypticase soy agar (TSA, BD) supplemented with 3% of sheep blood. For selection, 300 µg/ml of kanamycin and 1 mg/plate of Xgal were added.

2. DNA manipulation and plasmid constructions

DNA preparation, amplification, digestion, ligation and purification were performed according to standard techniques. Southern blot analyses were performed following manufacturer instruction (NEBlot phototop kit and Phototop star detection kit, New England Biolab). Primers used in this study were supplied by VBC-Biotech Services GmbH. Sequencing reactions were performed by VBC-Biotech Services GmbH.

3. Construction of *S. pyogenes* M1 SF370 *clpX*- and *clpP*-deficient strains

Deletion of *clpX* (Δ60-1161 bp), *clpP* (Δ1-591 bp), *clpE* (Δ1-2283 bp) and *clpC* (Δ115-2213 bp) were performed by temperature shift using a Gram-positive thermosensitive replicative plasmid pEC214. In the case of *clpX*, a fragment containing 955 bp upstream of the deleted region and a fragment containing 1069 bp downstream of the deleted region flanked by restriction enzyme were amplified and inserted into pEC214, leading to pEC231. In the case of *clpP*, a fragment containing 950 bp upstream of the deleted region and a fragment containing 1086 bp downstream of the deleted region flanked by restriction enzyme were amplified and inserted into pEC214, leading to pEC217. In the case of *clpE*, a fragment containing 947 bp upstream of the deleted region and a fragment containing 1054 bp downstream of the deleted region flanked by restriction enzyme were amplified and inserted

into pEC214, leading to pEC266. In the case of *clpC*, a fragment containing 457 bp upstream of the deleted region and a fragment containing 436 bp downstream of the deleted region flanked by restriction enzyme were amplified and inserted into pEC214, leading to pEC275. (Appendix Table 2 and 3). Wild-type *S. pyogenes* M1 SF370 electro-competent cells were transformed with the corresponding plasmid at 28°C selecting on kanamycin and Xgal. Positive clones were grown for 3 cycles at 37°C in the presence of kanamycin to induce the first recombination event. Clones were selected on kanamycin and Xgal. Integration of the plasmid was checked by PCR. Positive clones were grown for 3 cycles at 28°C without kanamycin to promote the second recombination event. White clones were selected and picked on TSA blood plates with and without kanamycin. Sensitive clones were analysed by PCR (Appendix Table 1). The deletion of *clpX*, *clpP*, *clpE* and *clpC* was verified by PCR, sequencing of the region and Southern blot analysis. The loss of the plasmid was ensured by temperature shift assays.

4. Complementation plasmids

The putative promoter region, the coding sequence and the putative terminator of *clpP*, *clpX* and *clpE* were amplified by PCR, flanked by restriction enzyme and inserted into pEC85, a Gram-positive replicative plasmid containing a kanamycine resistance cassette (Appendix Table 2). Plasmids were inserted into the corresponding competent mutants. pEC85 was used as transformation control. Cells were selected on kanamycin agar blood plate. Unfortunatly, no clone were retrieved in the case of the Δ*clpX* and Δ*clpP* with pEC85 or their corresponding plasmids.

5. Growth curves

For each strain, a THY overnight culture was diluted 1/100 into THY and grown at 37°C, 5% CO_2, 41°C, 5% CO_2 or at 37°C with agitation at 120 rpm. The OD_{620nm} was measured every hour.

6. Cfu analysis

For each strain, a THY overnight culture was diluted 1/100 into THY and grown at 37°C, 5% CO_2. Every hour, aliquots were removed and appropriate dilutions were plated in triplicates on TSA blood plate to determine the colony forming unit (cfu).

7. Survival under oxidative stress

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2 until mid-exponential phase. An aliquot was removed and H_2O_2 (Sigma) was added to the culture to a final concentration of 4 mM. After 1 h, 2 h and 3 h aliquots were removed and 0.05 mg/ml final concentration of catalase (Sigma) was added to stop the reaction. Appropriate dilutions were plated in triplicates on TSA blood plate to determine the colony forming units (cfu). The percentage of survival was obtained by the ratio of cfu / ml after stress to the cfu / ml prior to stress.

8. Survival upon lethal temperature

For each strain, a THY overnight culture was diluted 1/100 in THY and at 37°C, 5% CO_2 until mid-exponential phase. An aliquot was removed and the culture was transfered to 44°C, 5% CO_2. After 1 h, 2 h and 3 h aliquots were removed and appropriate dilutions were plated in triplicates on TSA blood plate to determine the cfu. The percentage of survival was obtained by the ratio of cfu / ml after stress to the cfu / ml prior to stress.

9. Osmotic stress

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2 until mid-exponential phase. 1 µl of dilutions 1 to 10^{-3} were spotted on a TSA blood plate supplemented with 0.65 M NaCl. Plate was incubated at 37°C, 5% CO_2 for 24 h and 48 h.

10. Penicilin resistance

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2 until stationary phase. After a dilution 1/100 into fresh THY, an aliquot was removed

and a final concentration of 100 µg/ml penicillin was added to the culture. After 2 h incubation at 37°C, 5% CO_2, appropriate dilutions of the culture were plated in triplicates onto TSA blood plate to determine the cfu.

11. Electron microscopy

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2 until early-stationary phase. Final concentrations of 2.5% paraformaldehyde, 0.015% glutaraldehyde and 30 mM phosphate buffer pH 7.0 were added to an aliquot of the culture. Cells were incubated 15 min at room temperature then transfered on ice for 30 min. After washing, cells were resuspended in 1x PBS and an aliquot was laid on L-polylysine coated slides. Slides were dried 30 min at 37°C, washed in 1x PBS and dipped 10 sec in methanol. Samples were treated 1 h with 1% osmium in 0.1 M cacodylate buffer pH 7.4. After 3 washings in ddH_2O, samples were dehydratated in a graded series of acetone solutions and critical point dried with hexamethyldisilazane (Merck). Samples were then covered with a 10 nm thick gold film and examined with a Fei XL30 scanning electron microscope.

12. Biofilm formation

For each strain, a C-medium overnight culture was diluted 1/10 in C-medium and 1.5 ml were inoculated in a 24 well plate (3 wells per strain). The plate was incubated at 37°C, 5% CO_2 for 24 h. The medium was removed and wells were washed gently two times with dH_2O. Adherent bacteria were stained with a solution of 0.2% crystal violet for 10 min at room temperature. Wells were washed gently with dH_2O. Adherent cells were retrieved with 1% SDS and the cell density was determined at OD_{540nm}.

13. Aggregation

Strains were grown overnight in THY under agitation (160 rpm) at 37°C. Cultures were kept at 37°C without agitation. The sedimentation rates were determined by the cell density of the upper layer of the culture measured at OD_{620nm}.

14. Adhesion to epithelial cells

HepA II cells were routinely grown in RPMI (Gibco) supplemented with 10% FCS (Sigma) and 1% penstrept (Sigma). Cells were regularly checked for mycoplasma infection. Prior to the experiment, HepA II cells were seeded in RPMI, 10% FCS without antibiotic, at 1.5 x 10^5 /well in 24 well plates and incubated for 24 h at 37°C, 5% CO_2. In parallel, bacterial strains from a THY overnight culture in were diluted 1/25 in THY and grown at 37°C, 5% CO_2 until stationary phase. Bacteria were harvested, washed with RPMI, 10% FCS and added to the HepAII at a multiplicity of infection (MOI) of 5. After 30 min incubation at 37°C, 5% CO_2, cells were washed five times with 1 x PBS to remove the non-adherent bacteria and then incubated 15 min in ice-cold dH_2O to retrieve adherent epithelial cells. Appropriate dilutions were plated to determine the colony forming units (cfu) per ml.

15. Northern blot analysis

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2. Cells were harvested at appropriate time points corresponding to OD_{620nm} (Lag: 0.08; EL: 0.2; ML: 0.32; LL: 0.44; ES: 0.5). Total RNA extracts were isolated using Trizol reagent (Invitrogen). 25 µg of RNA were separated on a formaldehyde agarose gel and transferred onto a Nylon N+ Hybond membrane (Amersham). PCR generated probes, corresponding to an internal fragment of genes, were labelled using α-^{32}P-dATP (Appendix Table 4).

For the heat-shock response, 2 cultures of EC904 were grown until late-exponential phase at 37°C, 5% CO_2. One of the cultures was transferred at 41°C, 5% CO_2. Aliquots of 10 ml of culture were harvested after 15, 30, 45 and 60 min.

16. Exoprotein purification and Western blot analysis

For each strain, a THY overnight culture was diluted 1/100 in THY and grown at 37°C, 5% CO_2. 20 ml of culture were harvested at appropriate time points corresponding to OD_{620nm} (EL: 0.2; ML: 0.32; LL: 0.44; ES: 0.5). Supernatants of culture were filtered and the proteins were precipitated using 10% TCA.

Exoproteins were separated by 8-18% gradient SDS-PAGE. Gels were fixed 3 h into a 40% ethanol, 10% acetic acid solution and transferred over night into a 0.1% coomassie G-250 solution.

For Western blot, proteins were transferred onto nitrocellulose membranes (Protan). Antibodies against SpeB (1/2000), M (1/1000), Sic (1/1000) and Ska (1/500) were diluted into 2% BSA, 1x TBST. Anti-rabbit (SpeB, Ska, Sic) and anti-mouse (M) HRP antibodies (Amersham) were diluted 1/10000 into TBST. Finally, the HRP activity was detected by chemoluminescent reagent (Pierce).

Results

1. Genetic organization of the Clp encoding genes

The genomic organization of the protease coding gene *clpP* and genes coding for the Clp ATPases participating in the Clp mediated proteolysis *clpX*, *clpE* and *clpC* loci in *S. pyogenes* are shown in Fig. 4. The *clpX* gene is located directly upstream of *yihA*, which encodes a homologue of YsxC, a GTP-binding protein essential for ribosome assembly and stability in *B. subtilis* and *S. aureus* (Fig. 4C) [320-321]. The *clpP* gene is located immediately downstream of the *upp* gene encoding a homologue of uracil phosphoribosyltransferase (Fig 4B) [322]. *clpC* is situated directly downstream of *ctsR*, encoding the negative transcriptional regulator of several *clp* genes in *B. subtilis* (Fig. 4E) [80]. Finally, *clpE* is located upstream of an orthologue of the mutator protein coding gene, *mutT* (Fig 4D) [323]. The tandem organization of *upp-clpP, clpX-yihA, ctsR-clpC* is conserved among *S. pyogenes* genomes and is also found in other streptococcal species, i.e. *S. mutans* and *S. pneumoniae* (Fig. 5) [324]. Interestingly in *B. subtilis*, *L. monocytogenes* and *S. aureus*, *clpC* is part of a *ctsR* regulated operon composed of *ctsR* itself, *mcsA*, *mcsB* and *clpC* (Fig. 5C) [80, 85]. In *S. pyogenes*, no homologues of *mcsA* or *mcsB* have been identified. Similar to other Gram-positive bacteria, the promoter regions of *clpP*, *clpE* and the *clpC* operon but not of *clpX* exhibit an heptanucleotide repeat motif (TTTGACC/T-$N_{(3)}$-TTTGACC/T) corresponding to a CtsR binding site (Fig. 4). Thus, it appears that the co-transcription of *ctsR* and *clpC*, regulated by CtsR itself is conserved among Gram-positive bacteria.

Furthermore, nucleotide sequences of *clpE*, *clpC*, *clpX* and *clpP* are 100% identical in all *S. pyogenes* genomes. *S. pyogenes* Clp proteins show a high percentage amino-acid sequence homology with the corresponding proteins in other Gram-positive species, with the highest score observed among streptococcal proteins (Fig. 6). *S. pyogenes* Clp ATPases ClpX, ClpC and ClpE contain the typical domains for ATPase activity, and domains involved in substrate and ClpP binding. The Walker A and Walker B motifs involved in the binding and hydrolysis of ATP, the P-loop domain required for the interaction with ClpP and the pore forming loop domain described in *B. subtilis* and other Gram-positive Clp ATPases are conserved (appendix Fig. S1,S3,S4) [15]. In addition, the zinc binding domain of ClpX involved in the binding of the substrate is also conserved in *S. pyogenes*. The catalytic triade

(SHD) essential for ClpP activity is also present and conserved in *S. pyogenes* and various Gram-positive bacteria (appendix Fig. S2).

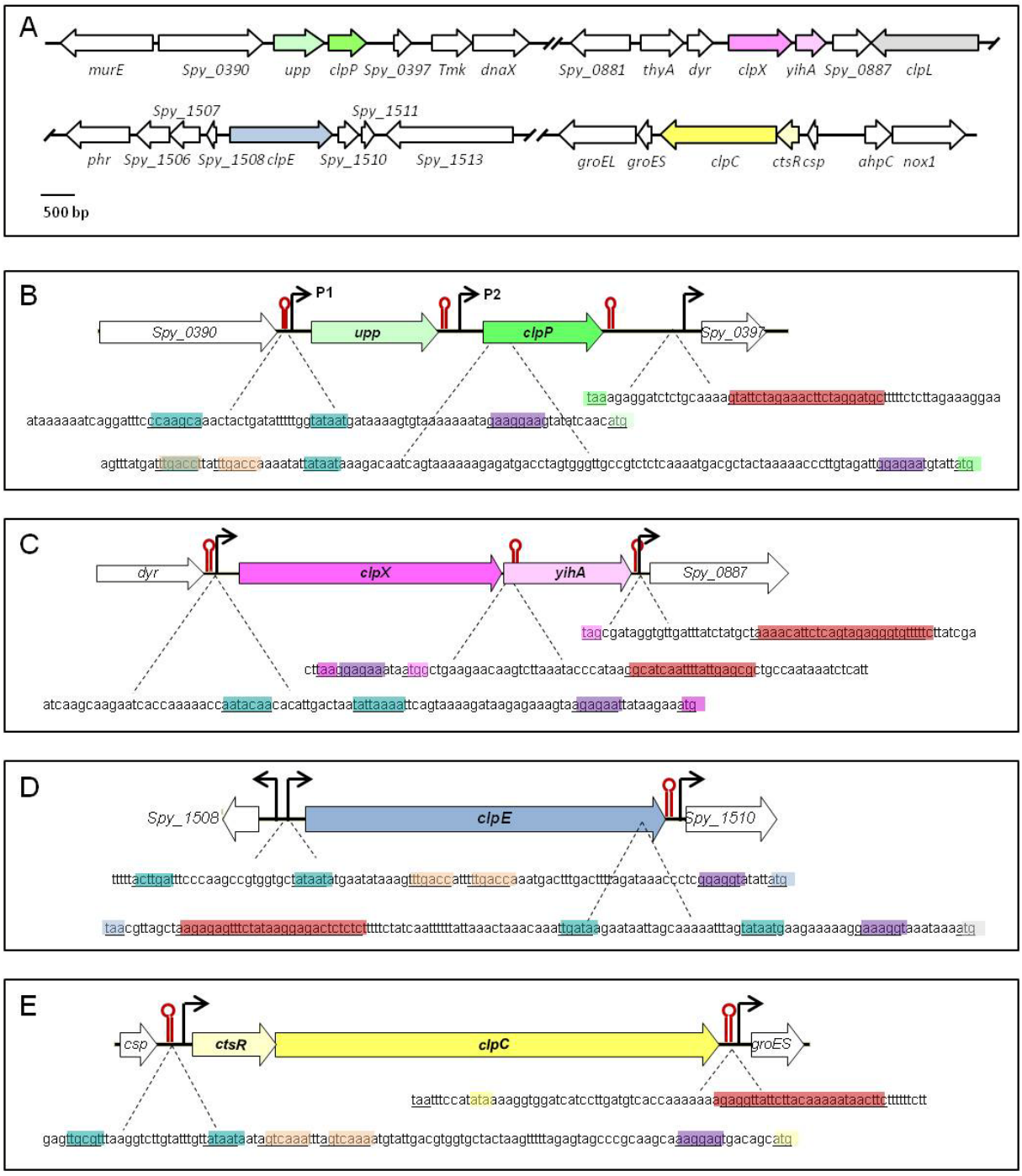


Fig. 4. Genomic organisation of the *clp* loci in *S. pyogenes*. (A). Gene sequences surrounding *clpP* (green), *clpX* (pink), *clpE* (blue) and *clpC* (yellow) are depicted to scale. (B-E). Prediction of the promoter and terminator region of *clpP* (B), *clpX* (C), *clpE* (D) and *clpC* (E). -35 and -10 box are highlighted in green, the RBS sequence in purple, the CtsR binding site in orange and the Rho-independent terminator in red.

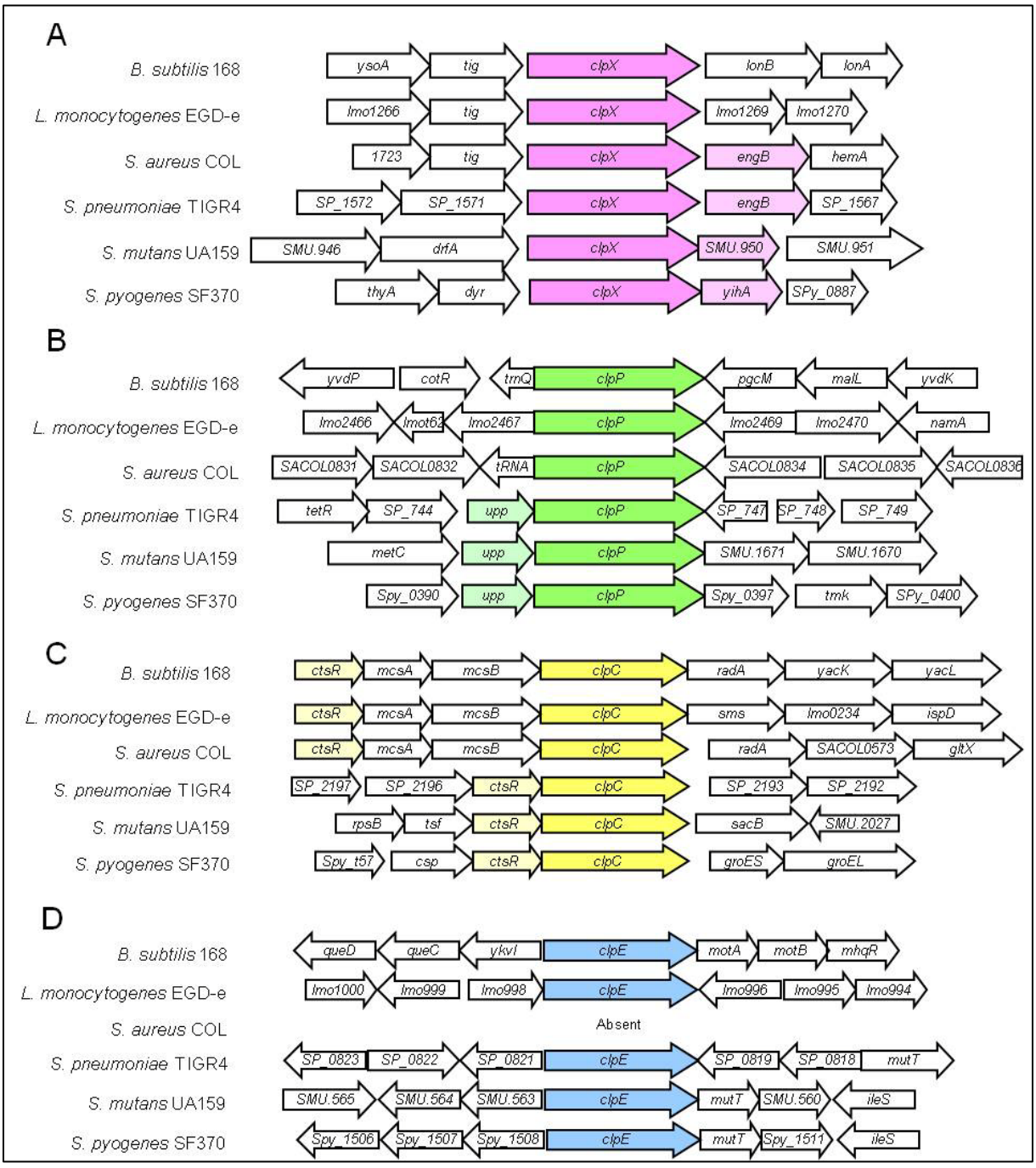


Fig. 5. Genomic organisation of the *clp* loci in Gram-positive bacteria. Region organisation surrounding *clpX* (A), *clpP* (B), *clpC* (C) and *clpE* (D). The genes are not depicted to scale. The nature of the genes stated are listed in the annexe.

2. Construction *clp*-deficient strains

To determine the impact of the Clp proteins in *S. pyogenes,* each of the respective genes were deleted from the chromosome. Because of the genomic arrangement of *clpE*, *clpC*, *clpX* and *clpP*, in-frame deletion mutants with non-polar effect on the neighboring genes were constructed. As wild-type strain, we chose *S. pyogenes* SF370, a reference clinical isolate of M1 serotype which complete genome sequence is publicly available (accession number: ATCC 700294). The *S. pyogenes* M1 type is the most representative serotype among clinical isolates world-wide and is linked to minor infections including pharyngitis but also to lethal infections such as toxic shock syndrome.

Percentage identiy of the aa sequence	*S. pyogenes*			
	clpC	*clpE*	*clpP*	*clpX*
B. subtilis	42	56	56	63
L. monocytogenes	43	59	60	66
S. aureus	38	-	60	65
S. pneumoniae	65	81	90	82
S. mutans	85	90	85	86

Fig. 6. Homology of Clp proteins in Gram-positive bacteria. Percentage of amino-acid sequence identity of Clp proteins between *S. pyogenes* SF370, and *B. subtilis* 168, *L. monocytogenes* EGD-e, *S. aureus* COL, *S. pneumoniae* TIGR4 and *S. mutans* UA159.

For *clpP and clpE,* the entire coding sequences of 591 nt and 2283 nt, respectively, were deleted in-frame. However, in the case of *clpX* only an internal fragment of 1101 bp (60 - 1161) was deleted in-frame in order to keep the *yihA* ribosome binding site located at the 3' end of the coding sequence of *clpX* in the *clpX*-deficient strain. Because of the tandem transcriptional organization of *clpC* and *ctsR*, only an internal fragment of *clpC* of 2098 nt (Δ115-2213) was deleted from the chromosome. Thus, in both generated Δ*clpX* and Δ*clpC* mutants, expression of the downstream co-transcribed gene should be unaffected compared to the wild-type.

The upstream and downstream regions surrounding the sequence to delete were cloned into a thermosensitive plasmid, pEC214 (Appendix Table 2). Following enzymatic restriction and sequence analysis, the plasmids were introduced into *S. pyogenes* M1 SF370. Then, strains underwent several temperature shifts promoting recombination events that ultimately led to the generation of Δ*clp* mutants or wild-type strains. Wild-type strains resulting from

this procedure are refered to as “WTts” in this study. They were kept and used in the study as controls in the analysis of Δ*clp* phenotypes (Appendix Table 1).

After the deficient strains were obtained, complementation plasmids were constructed by inserting the coding sequence as well as the putative promoter region and the putative transcriptional terminator of each *clp* gene into pEC85 (Appendix Table 2). The complementation plasmids were successfully introduced into the wild-type strain and the Δ*clpE* mutant. However, attempts to transform Δ*clpX* and Δ*clpP* mutants with the corresponding complementation plasmids or pEC85 failed. Therefore, no complemented strains for Δ*clpX* and Δ*clpP* could be obtained. Introduction of the *clpC* complementation plasmid into the Δ*clpC* mutant remains to be performed.

3. Analysis of the *clpX*- and *clpP*-deficient strains

3.1. Transcriptional analysis of *clpX* and *clpP* mutants

Sequence analysis of the *clpX-yihA* region revealed the presence of a putative promoter located 40 bp upstream of the translational start codon (Fig. 4C). We predicted two putative Rho-independent transcriptional terminators, located in the *clpX-yihA* intergenic region and downstream of *yihA*. Accordingly, Northern blot analysis of *clpX* shows expression of two transcripts of approximately 1300 nt and 1900 nt, that correspond in size to the predicted transcripts, starting from the same transcription start but stopping at two distinct Rho-independent terminators (Fig. 7). Expression analysis throughout the growth phase shows a decreased amount of both *clpX* transcripts when cells enter stationary phase. In addition, *clpX* expression is up-regulated in the Δ*clpP* mutant compared to the wild-type cultures from lag to late-logarithmic phase (Fig. 7). In *B. subtilis*, expression of *clpX* is not regulated by CtsR [92]. The absence of a CtsR binding site in the promoter region of *clpX* in *S. pyogenes* confirms that *clpX* is likely to not be directly regulated by CtsR in this organism. In the case of *upp-clpP*, we identified two putative promoters upstream of *upp* (P1) and in the *upp-clpP* intergenic region (P2) (Fig. 4B). Only one putative Rho-independent transcriptional terminator located downstream of *clpP* was identified. Consistent with sequence predictions, two distinct *clpP*-specific transcripts were detected by Northern blot analysis (Fig. 7).

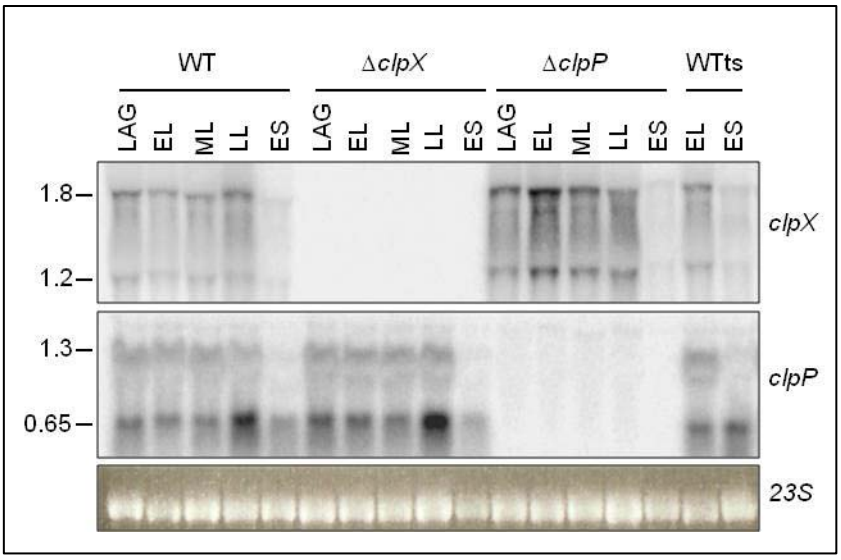


Fig. 7. Transcriptional analysis of *clpX* and *clpP* expression. Northern blot analysis of wild-type (WT), Δ*clpX*, Δ*clpP* and control wild-type (WTts) total RNA prepared throughout growth: LAG (lag phase), EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). Transcripts were detected with *clpX* or *clpP* specific PCR generated probes labeled with α-^{32}P-dATP. *clpX* and *clpP* are expressed both as mono- and bi-cistronic transcripts**.** The estimated sizes of the transcripts are indicated in Kilo-bases. 23S rRNA was used as a loading control.

Constitutive expression of both transcripts is observed from lag- to mid-logarithmic phase. Stronger expression of the shorter transcript in comparison to the longer transcript is observed at late-logarithmic and early-stationary phase. Expression of the longer transcript is also decreased when cells enter stationary phase. These data indicate that at late growth phase, activity of the promoter located upstream of *clpP* is enhanced compared to that located upstream of *upp.* Interestingly, a CtsR binding is located in the intergenic region between *upp* and *clpP*, overlapping the promoter region P2. As Clp ATPases and proteases are qualified as heat-shock proteins, we analyzed the influence of heat on *S. pyogenes clpX* and *clpP* expression. Northern blot analysis shows that shifting *S. pyogenes* cultures from 37°C to 41°C induced an increase of expression of the shorter transcripts of *clpX* after 30 min and *clpP* after 15 min (Fig. 8). Thus, *clpX* and *clpP* are induced by heat and both transcribed as mono- and bi-cistronic transcripts. Moreover, the regulation of *clpX* and *clpP* expression by heat is mediated via the mono-cistronic transcript.

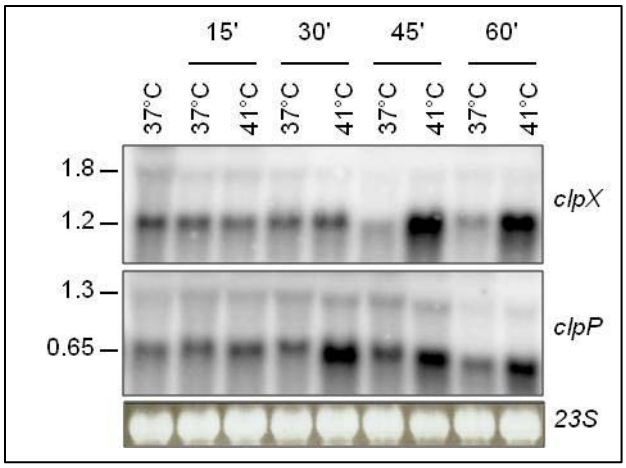


Fig. 8. Heat inducible expression of *clpX*- and *clpP-deficient strains.* Northern blot analysis of total RNA harvested from wild-type culture after 15, 30, 45 and 60 min of heat shock at 41°C. Transcripts were detected with *clpX*- or *clpP*- specific PCR generated probes labeled with α-^{32}P-dATP. *clpP* expression is induced by heat after 15 min whereas *clpX* expression is induced after 30 min. The estimated sizes of the transcripts are indicated in Kilobases. 23S rRNA was used as a loading control.

3.2. Growth of Δ*clpX* and Δ*clpP* mutants

Growing cells in THY medium, at 37°C, 5% CO_2 revealed a slower growth characterized by a longer lag phase of Δ*clpX* and Δ*clpP* mutants compared to the wild-type parent (Fig. 9A). Moreover, both mutants failed to reach the same density of cells at stationary phase compared to the wild type.

As a more direct read-out of cell viability of Δ*clpX* and Δ*clpP* mutants, colony forming units (Cfu) were determined throughout the growth curve (Fig. 9B). After 1 h of growth, the wild-type already started duplicating whereas at this time point the number of Δ*clpX* and Δ*clpP* cells remained unchanged. This result may provide an explanation for the longer lag phase and the slight delay of growth observed in the mutants. During exponential phase, the generation time was similar comparing the wild-type and Δ*clp* mutant strains (Fig. 9B). Interestingly, at stationary phase, even when the density of cells was lower for Δ*clpX* and Δ*clpP,* the number of cells was equal to the wild-type strain. To conclude, Δ*clpX* and Δ*clpP* mutant strains exhibit a longer lag phase explaining their delay of growth compared to the wild-type. However, the generation time and number of cells at stationary phase of Δ*clpX* and Δ*clpP* mutants is comparable to the ones of the wild-type whereas the cell density is decreased which could be explained by the decreased size of the Δ*clpX* and Δ*clpP* mutant cells.

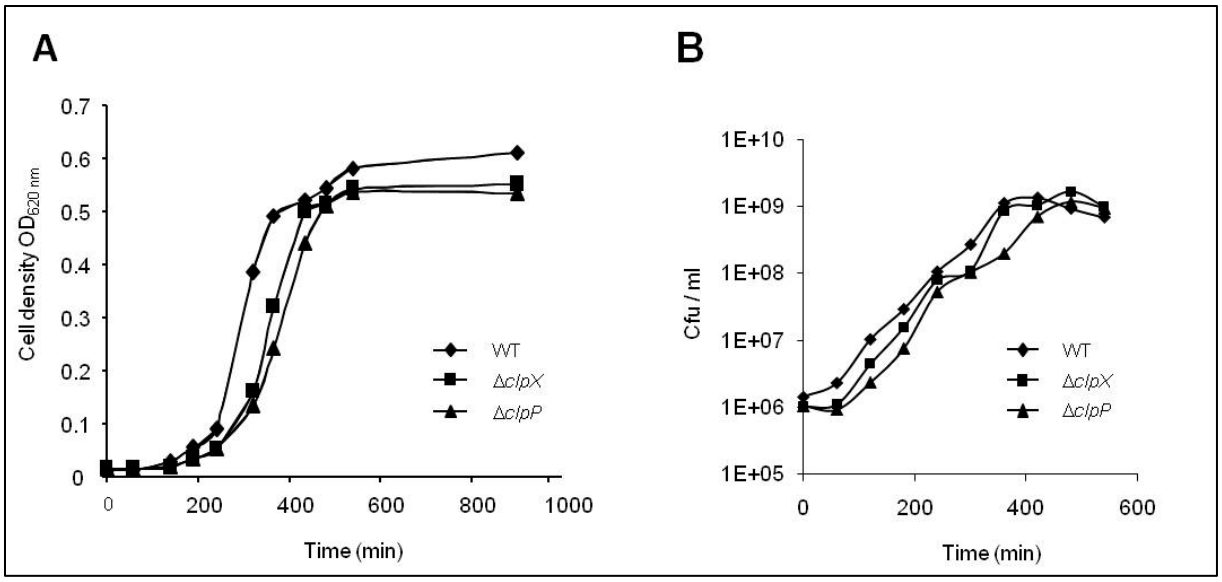


Fig. 9. Growth of *clpX- and clpP-deficient* strains is delayed due to a longer lag phase. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated in THY at 37°C, 5% CO_2. (A) Culture density was measured regularly at $OD_{620\ nm}$. The results from three independent cultures are expressed as mean $OD_{620\ nm}$. (B) Aliquots of cultures were removed during growth and colony forming units (cfu) / ml were determined by plating dilution series onto TSA blood in triplicates. The experiment was performed three times independently. A representative result is shown.

3.3. Cell morphology analysis

3.3.1. Biofilm formation

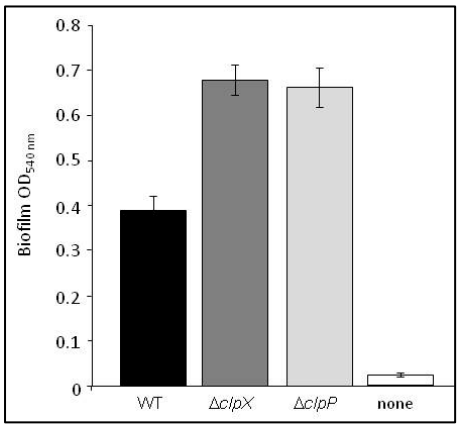


Fig. 10. ClpP and ClpX are involved in biofilm formation. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated in triplicates for 24 h in C-medium at 37°C, 5% CO_2. After washing with PBS, attached cells were dyed with 0.2% crystal violet. Cells were retrieved with 1% SDS solution and cell density was determined at $OD_{540\ nm}$. The values shown are the average of three inocula ± SD. The experiment was performed three times independently. A representative result is shown

As a defense mechanism against the host, *S. pyogenes* is able to grow in biofilms [325]. Here, the capacity of the *clpX*- and *clpP*-deficient strains to form biofilm in rich medium (C-medium) was evaluated. After 24 h, Δ*clpX* and Δ*clpP* showed a significant increase in biofilm formation on polystyrene plastic surfaces (Fig. 10). Quantifications revealed that Δ*clpX* and Δ*clpP* produced approximately twice more biofilm than the parent strain SF370. The same results were observed after 48 h incubation at 37°C, 5% CO_2.

3.3.2. Aggregation

During culture in rich medium (THY) at 37°C without agitation, Δ*clpX* and Δ*clpP* were observed to settle down earlier than the wild-type. Consequently, sedimentation was evaluated by measuring the $OD_{620\ nm}$ of the upper layer of culture after growth under agitation. Our measurements showed that after 4 h, the wild-type strain reached the $OD_{620\ nm}$ of 0.3 (Fig. 11). In the case of Δ*clpX* and Δ*clpP*, the $OD_{620\ nm}$ of 0.3 was already reached after 1 h and no cells were left in the upper layer after 4 h without agitation. This result shows that the *clpX*-and *clpP*-deficient strains are forming more aggregates than the parent strain SF370.

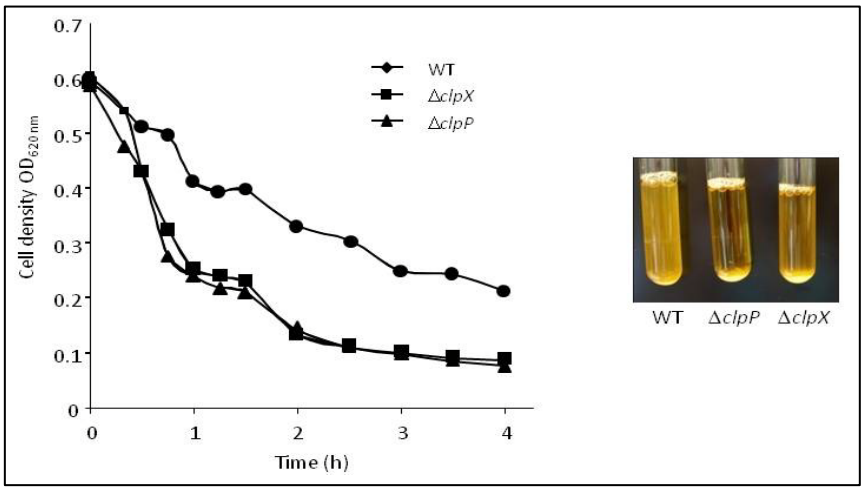


Fig. 11. *clpX* and *clpP* deletions affect streptococcal aggregation. Wild-type (WT), Δ *clpX* and Δ *clpP* were cultivated over-night in THY medium at 37°C with shaking. Culture were kept standing. Cell density of the upper phase of culture was determined at $OD_{620\ nm}$. The picture shows cultures standing after one hour. The experiment was performed three times independently. A representative result is shown.

3.3.3. Microscopy

S. pyogenes has the characteristic to grow in pairs of cells or in chains of varying lengths [326]. A defect in chain formation could explain the increase in aggregation of the Δ*clpX* and Δ*clpP* strains. Stationary phase cultures of wild-type, Δ*clpX* and Δ*clpP* were observed by scanning electron microscopy. Compare to wild-type, Δ*clpX* and Δ*clpP* exhibited longer chains of diplococci (Fig. 12 upper panel). In addition, chains of the Δ*clpP* mutant were disorganized and aggregated with cells that appeared to not properly segregate. It appeared as well that Δ*clpP* cells were slightly smaller than Δ*clpX* or the parent strain (Fig. 12 lower panel). As predicted above, the decrease in cell size of the mutants could explain their reduced cell density at stationary phase. In conclusion, these results suggest that ClpX and

ClpP are involved in biofilm formation and cell aggregation. In addition, ClpP seems to affect cell segregation.

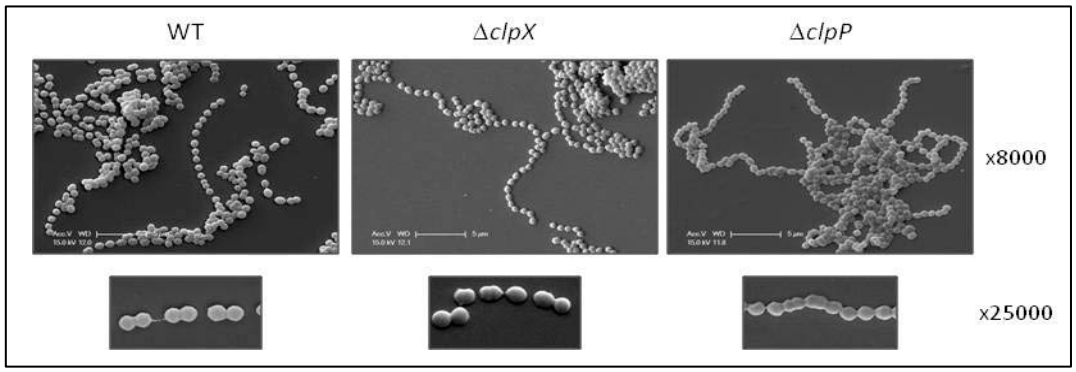


Fig. 12. Scanning electron microscopy reveals that *clpX* and *clpP* deletion affect chain formation. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated until mid-log phase. Cells were fixed to poly-lysine coated coverslips and dried as explained in « Materials and Methods ». Samples were covered with a 10 nm thick gold film and examined with a Fei XL30 scanning electron microscope. Chains were observed at magnification x 8 000 (upper panel) and x 25 000 (lower panel).

3.4. Tolerance to stress conditions

3.4.1. Growth under stress conditions

In several other Gram-positive bacteria, ClpX and ClpP are involved in stress response. First, tolerance to heat and oxygen was assessed by monitoring the growth of wild-type, Δ*clpX* and Δ*clpP* strains in rich medium at 41°C and at 37°C under shaking, respectively (Fig. 13).

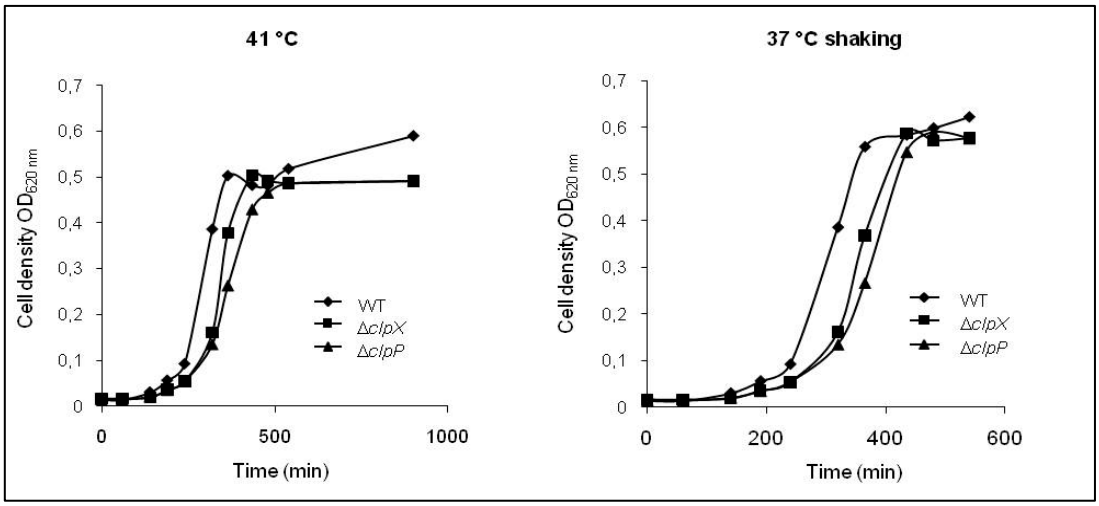


Fig. 13. Growth of *clpX- and clpP-deficient* strains is not affected at 41°C or with oxygen. Wild-type (WT), Δ*clpX* and Δ*clpP* strains were cultivated in THY medium at 41°C, 5% CO_2 (A) or at 37°C under agitation (B). Culture density was measured regularly at $OD_{620\ nm}$. The experiment was performed three times independently. A representative result is shown.

Interestingly, Δ*clpX* and Δ*clpP* mutants exhibited a delay of growth compared to the wild-type. In the two stresses tested, the delay in growth was comparable to the one already observed at 37°C, 5% CO_2. Consequently, ClpX and ClpP are not involved in the response to heat-shock or oxygen exposure.

3.4.2. Survival under critical conditions

Additionally, we assessed the survival of Δ*clpX* and Δ*clpP* mutants under the following critical conditions: lethal temperature, superoxide compounds, high salt concentration and acidic pH.

Surprisingly, the deletion of *clpX* or *clpP* provided an advantage for *S. pyogenes* to tolerate lethal temperature (Fig. 14A). Compared to wild-type, the Δ*clpX* and Δ*clpP* mutants showed an increased survival rate after shifting the cultures from 37°C to 44°C over a 3 hour period.

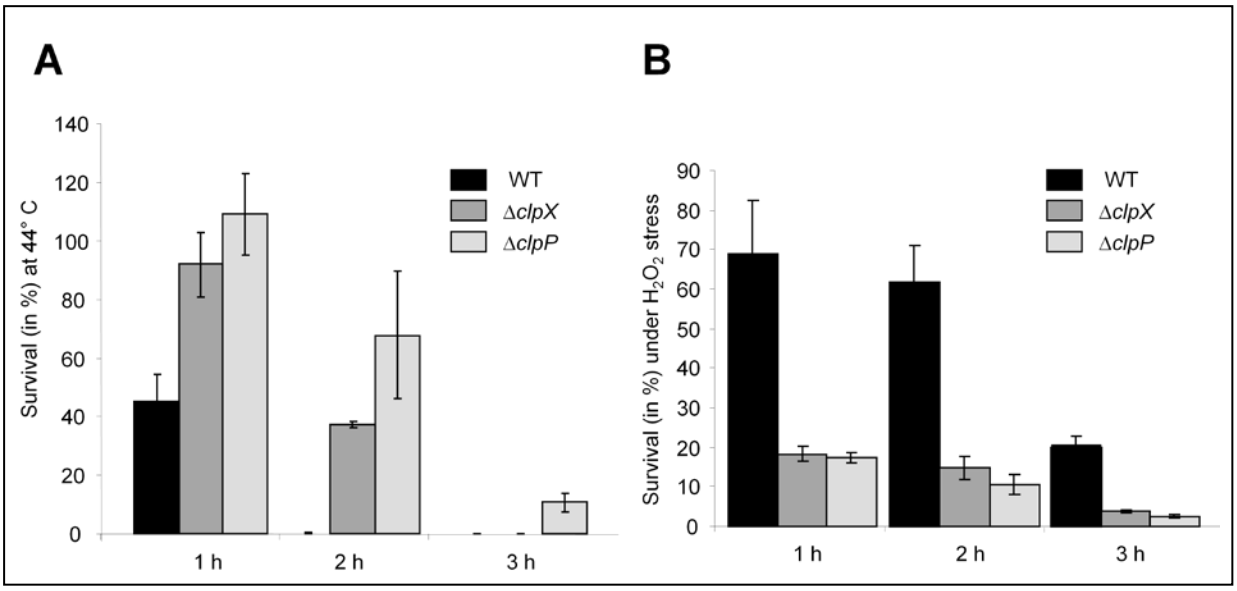


Fig. 14. ClpX and ClpP are involved in the response to heat-shock and oxidative stress. (A) Resistance to lethal temperature. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated in THY at 37°C, 5% CO_2. At mid-log phase, culture temperature was shifted to 44°C. Aliquots were collected every hour and the ratio of colony forming units (cfu) / ml was determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. The experiment was performed three times independently. A representative result is shown. (B) Resistance to oxidative stress. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated in THY medium at 37°C, 5% CO_2. At mid-log phase, 4 mM H_2O_2 were added to the culture. Aliquots were collected every hour and colony forming units (cfu) / ml were determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. The experiment was performed three times independently. A representative result is shown.

In contrast, *clpX*- and *clpP*-deficient strains were more sensitive to exposure to 4 mM H_2O_2 than the wild-type (Fig. 14B). After 1 h, only 20% of Δ*clpX* and Δ*clpP* cells survived compared to 70% for the parental strain. The ratio remained constant over a time period of 3 hours.

Furthermore, resistance to high salt concentration was examined. Cells were grown on plate containing 0.65 M NaCl. After 24 h incubation, wild-type and Δ*clpX* exhibited colonies until the dilution 10^{-4} whereas Δ*clpP* displayed a decrease in resistance of about 3-log with colonies observed until 10^{-1} dilution (Fig. 15).

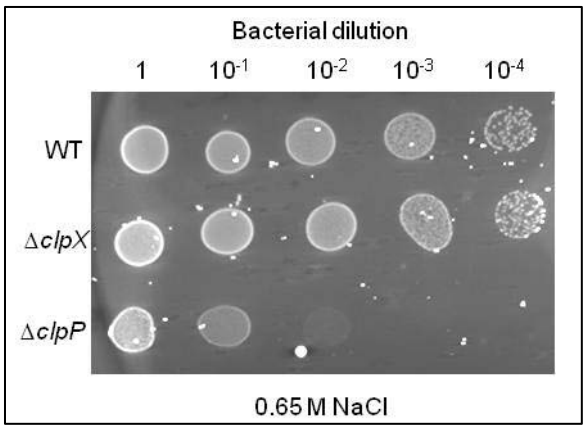


Fig. 15. ClpP but not ClpX is involved in the resistance to salt stress. Wild-type (WT), Δ*clpX* and Δ*clpP* were cultivated in THY medium at 37°C, 5% CO_2 until stationary phase. Serial dilutions were spotted onto a 0.65 M NaCl blood plate. After 24 h of growth at 37°C 5% CO_2 plate was scanned. The experiment was performed three times independently. A representative result is shown.

Finally, wild-type, Δ*clpX* and Δ*clpP* strains were subjected to acidic stress. Cells were incubated at pH 5.0 and pH 6.0 in PBS. No significant differences were observed compared to the wild-type strain (data not shown).

3.4.3. Antibiotic resistance

Penicillin is typically used to treat infections caused by *S. pyogenes* such as pharyngitis, necrotisis fasciitis and toxic shock syndrome [141]. In order to determine the role of ClpX and ClpP in penicillin resistance, the deficient strains were incubated for 2 h with a lethal concentration of penicillin (Fig. 16). Δ*clpX* and Δ*clpP* mutants were 10 times more resistant than the wild-type strain.

In conclusion, ClpX and ClpP are required for *S. pyogenes* to tolerate oxidative stress whereas they negatively regulate penicillin-mediated killing and resistance to heat-shock. Additionally, ClpP is necessary for resistance of *S. pyogenes* to high salt concentration.

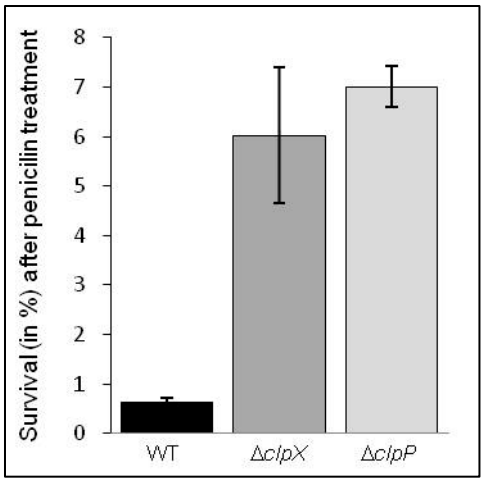


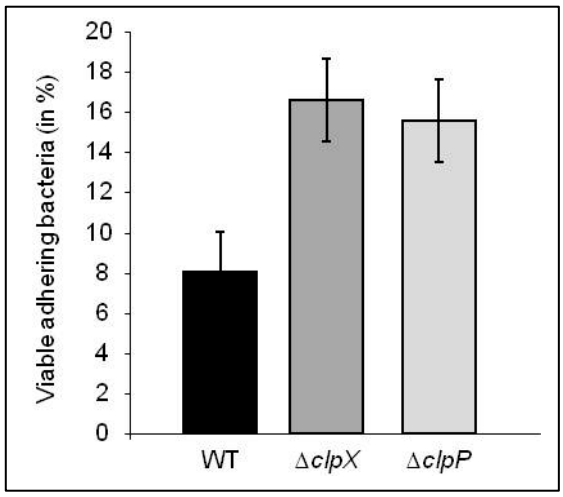
Fig. 16. *clpX* and *clpP* are involved in resistance to penicillin. Wild-type (WT), Δ*clpX* and Δ*clpP* strains were cultivated in THY medium at 37°C, 5% CO_2 until early stationary phase. 60 ng/ml final concentration of penicillin was added to the culture. After 2 hours incubation, cells were washed. Survival was determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. The experiment was performed three times independently. A representative result is shown.

3.5. Adhesion to epithelial cells

The enhanced aggregation observed in Δ*clpX* and Δ*clpP* mutants suggested a possible role of ClpX and ClpP in adhesion of *S. pyogenes* to epithelial cells. Interactions of wild-type, Δ*clpX* and Δ*clpP* strains with monolayers of human pharyngeal epithelial cells were analyzed (Fig. 17). With the mutant strains, a 2-fold increase of bacteria adhering to epithelial cells was observed after 30 min incubation, when compared to wild-type.

In addition, we analyzed the internalization by epithelial cells of the *clpX- and clpP-deficient* strains compared to the wild-type strain. Epithelial cells were incubated with Δ*clpX*, Δ*clpP* or wild-type strains for 1 h and treated with penicillin to kill the external remaining bacteria. However, as the Δ*clpX* and Δ*clpP* strains are more resistant to penicillin than the wild-type, our results were compromised.

Fig. 17. Deletion of *clpX* and *clpP* enhances the adhesion of *S. pyogenes* to epithelial cells. Wild-type (WT), Δ*clpX* and Δ*clpP* strains were grown until early stationary phase. Hep-2 II cells were infected with the GAS strains at a MOI of 5 in triplicates. After 30 min of incubation, epithelial cells were washed with PBS and adherent bacteria were retrieved. Survival was determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. The experiment was performed three times independently. A representative result is shown.



3.6. Exoprotein expression

The increased adhesion of Δ*clpX* and Δ*clpP* cells to epithelial cells indicated that ClpX and ClpP are involved in regulation of virulence factor expression. Consequently, the virulence factor expression pattern of Δ*clpX* and Δ*clpP* strains was analyzed. Exoproteins from *S. pyogenes* cultures at different time points during growth were prepared and analyzed by SDS-PAGE (Fig. 18). Striking differences in the exoprotein pattern between the wild-type and mutants were revealed throughout the growth. Six proteins displaying a different expression pattern in Δ*clpX* and Δ*clpP* mutants were extracted from the gel and analyzed by mass spectrometry. Five proteins were identified as M protein, streptokinase A (Ska), streptococcal inhibitor of complement (Sic), cysteine protease SpeB and superantigen SpeC. M protein, Ska and Sic amounts were enhanced in Δ*clpX* and Δ*clpP* mutants whereas SpeB and SpeC expression was reduced.

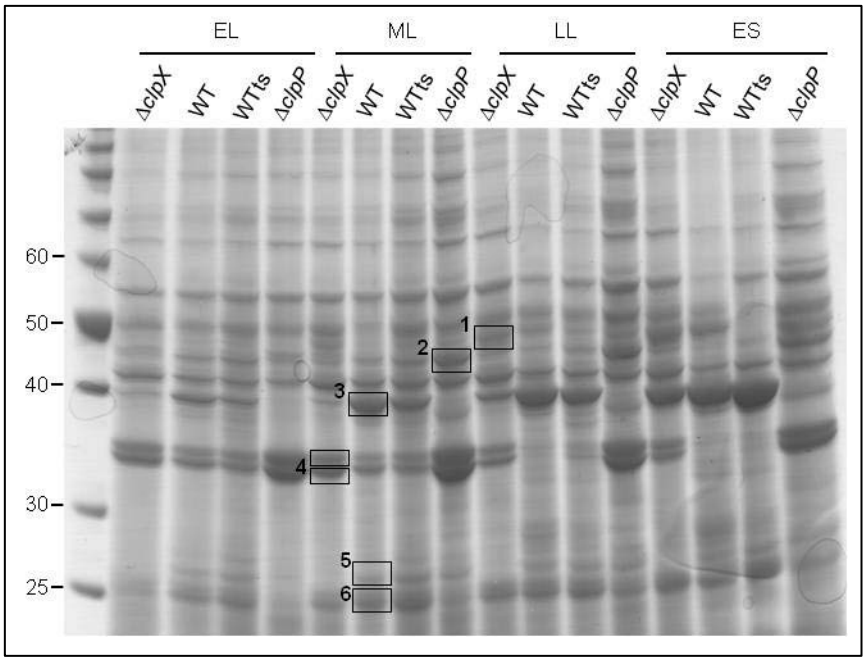


Fig. 18. Exoprotein expression pattern of *clpX*- and *clpP*-deficient strains. Wild-type (WT), Δ*clpX*, Δ*clpP* and control wild-type (WTts) exoproteins were harvested during growth: EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). Proteins were separated on a gradient (8-18%) SDS PAGE and were detected with colloidal comassie staining. The black boxes represent the proteins sent for identification by mass spectroscopy. (1) M protein, (2) Ska, (3 & 5) SpeB, (4) Sic and (6) SpeC were identified. The sizes of the ladder are indicated in kDa.

To verify the observed influence of ClpX and ClpP on virulence factor expression, western blot analysis using specific antibodies against M protein, Ska, Sic and SpeB were performed (Fig. 19). The results confirmed the mass spectrometry outcome. The expression of

M protein and Sic was constitutively up-regulated throughout growth. However, while Sic was not anymore expressed in the wild-type grown to late-log and early-stationary phase, its expression remained constant in the *clpX- and clpP-deficient* strains suggesting an increased stability in the mutants. In the case of SpeB, there was a total absence of expression in Δ*clpP*, whereas in Δ*clpX*, SpeB was expressed but not processed to its 28 kDa mature form. In conclusion, ClpX and ClpP are involved in the regulation of expression of SpeB, M protein, Ska and Sic but also in the maturation of SpeB.

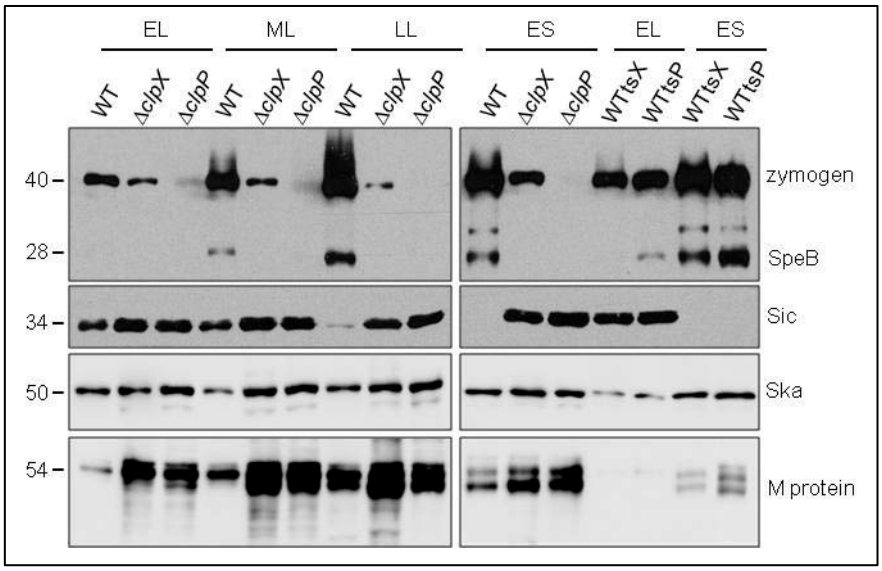


Fig. 19. Virulence factor expression analysis by Western blot. Western blot analysis of wild-type, Δ*clpX*, Δ*clpP* and control wild-type (WTts) exoproteins harvested during growth: EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). 5 µg of exoproteins were loaded on a 10% SDS-PAGE. Additionally, equal loading was determined by ponceau staining of the membrane. Proteins were detected with specific antibodies against SpeB, Ska, Sic and M protein. Sizes are indicated in kDa.

3.7. Transcriptional regulation

In order to determine if ClpP and ClpX act at the transcriptional level, expression of the main streptococcal virulence genes was studied by Northern blot analysis. Interestingly the gene expression profile was similar to the previously observed exoprotein profile (Fig. 20). An increased expression of the genes coding for Ska (*ska*), M protein (*emm*) and Sic (*sic*) was observed in the *clpX*- and *clpP*-deficient strains. The expression of SpeB encoding gene (*speB),* was clearly reduced in Δ*clpX* and abolished in Δ*clpP*. Morever the expression of *sagA* coding for Streptolysin S, *slo* coding for Steptolysin O and *speC* coding for Superantigen C was diminished in Δ*clpX* and Δ*clpP* mutants compared to the parental strain. In conclusion, ClpX and ClpP down-regulate the expression of *emm*, *ska* and *sic* and upregulate the

expression *of slo, sls, speC* and *speB*. This result illustrates that ClpX and ClpP regulate GAS virulence factor expression at the transcriptional level.

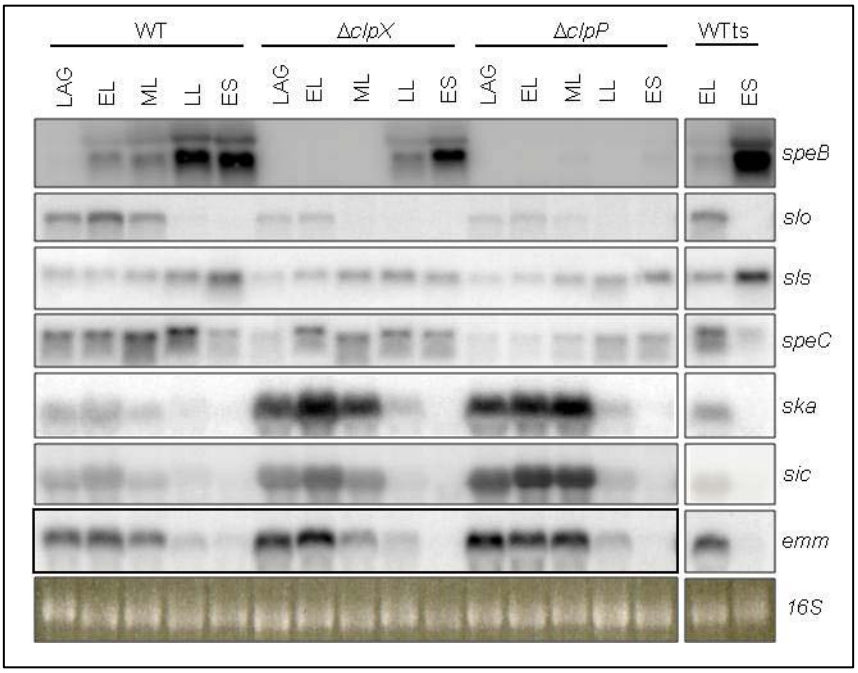


Fig. 20. Transcriptional analysis of *S. pyogenes* virulence factor expression. Northern blot analysis of wild-type (WT), Δ*clpX*, Δ*clpP* and control wild-type (WTts) total RNA harvested during growth: LAG (lag-phase), EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). Transcripts were detected using gene specific PCR generated probes labeled with α-^{32}P-dATP. *speB, slo, sls* and *speC* expression is down-regulated in the mutants whereas *ska, sic* and *emm* expression is up-regulated. 16S rRNA was used as a loading control.

3.8. Regulator expression

RopB is the main regulator of *speB* expression [265, 269]. *rgg*, the gene coding for RopB, is located adjacent to *speB* on the chromosome. As the expression of *speB* was diminished in the Δ*clpX* mutant and abolished in the Δ*clpP* mutant, the expression of *rgg* was examined. The mapping of the *rgg* promoter region by primer extension revealed 2 distinct promoters at -114 nt (P_1) and -922 nt (P_2) upstream the start of translation (Fig. 21) (unpublished data). Accordingly, *rgg* was expressed as two independent transcripts of approximately 1000 nt and 1800 nt, respectively (Fig. 22). Analyzing the expression of *rgg* throughout the growth, it appeared that during exponential phase, *rgg* was expressed via the promoter P_2. Then, later in the exponential phase, a switch of expression from P_2 to P_1 occurred leading to an expression via P_1 during stationary phase. In Δ*clpX* and Δ*clpP*, *rgg* expression from P_2 continued to late exponential phase and an abolition of *rgg* expression from P_1 was observed in late exponential and stationary phase.

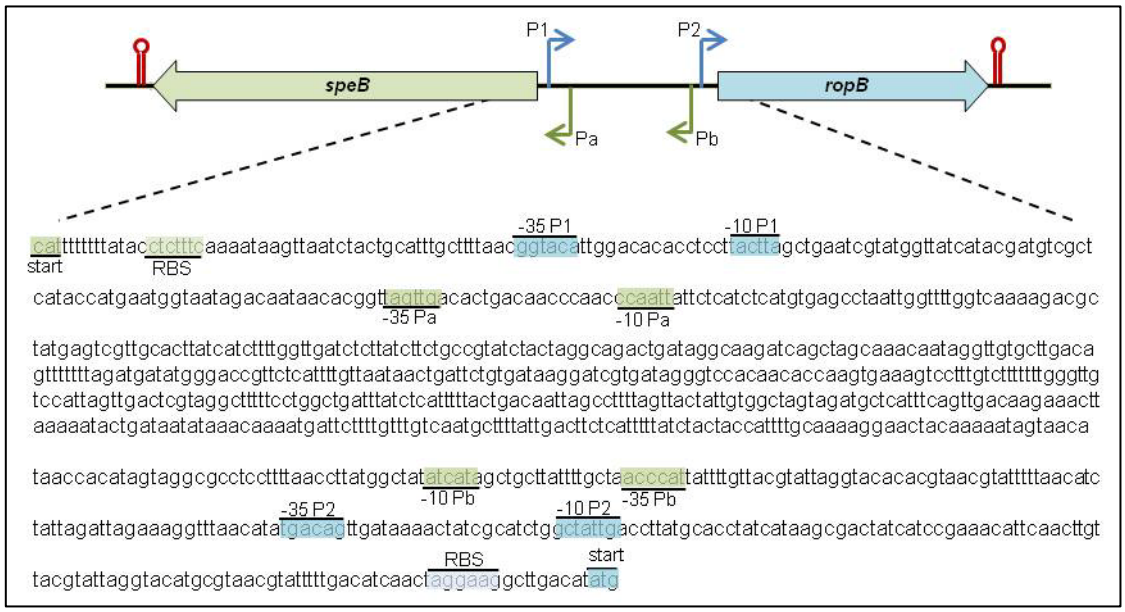


Fig. 21. Genomic organisation of the *speB-ropB* intergenic region. The promoter regions of *ropB* (P1 and P2) located by primer extension are indicated in blue. The promoter regions of *speB* (Pa and Pb) are highlighted in green [269]

To determine whether only *rgg* was affected by ClpX and ClpP, other streptococcal regulators were examined by Northern blot. First, the expression of genes encoding three other stand-alone regulators, *mga*, *nra* and *rofA*, did not seem to be affected by the deletion of *clpX* or *clpP*. Other regulators such as CodY, PerR, MtsR, MalR or SigX were studied and no difference in expression of the corresponding genes was observed (data not shown). However, ClpX and ClpP affected the expression of *covR* and *fasA* encoding the response regulators of the two-component systems CovRS and FasBCAX, respectively as well as the expression of *fasX*, a small non coding RNA regulating expression of the FasBCA operon (Fig. 22). In fact, the expression of *covR*, *fasA* and *fasX* was decreased in Δ*clpX* and Δ*clpP* mutants compared to the wild-type. To conclude, ClpX and ClpP positively regulate *in vivo* the expression of the transcriptional regulator RopB and the two-component systems, CovRS and FasBCAX.

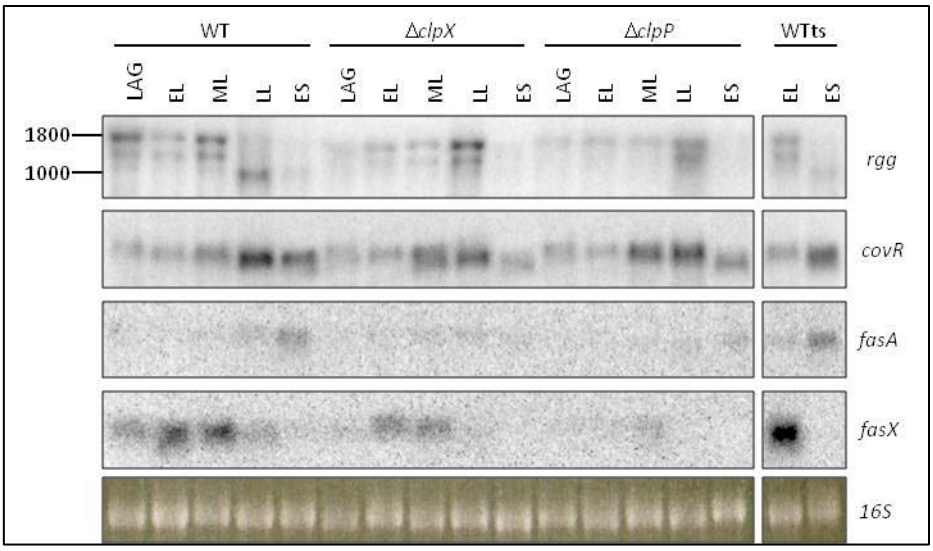


Fig. 22. Transcriptional analysis of *S. pyogenes* virulence regulator expression. Northern blot analysis of wild type (WT), Δ*clpX*, Δ*clpP* and control wild type (WTts) total RNA harvested during growth: LAG (lag phase), EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). Transcripts were detected using gene specific PCR generated probe labeled with α-^{32}P-dATP. Expression of *ropB, covR, fasA* and *fasX* is down-regulated in the mutants. 16S rRNA was used as a loading control. Size are indicated in nt

4. Analysis of *clpE*-deficient strain

4.1. Growth

Since *clpX*- and *clpP*-deficient strains exhibited a delay of growth most probably due to a longer lag-phase, it was interesting to study the growth of the *clpE*-deficient strain. The growth was monitored in rich medium at 37°C, 5% CO_2. Interestingly, Δ*clpE* displayed the same growth as the wild-type and complemented strain (Fig. 23). Therefore, we conclude that ClpE is not involved in the growth of *S. pyogenes*.

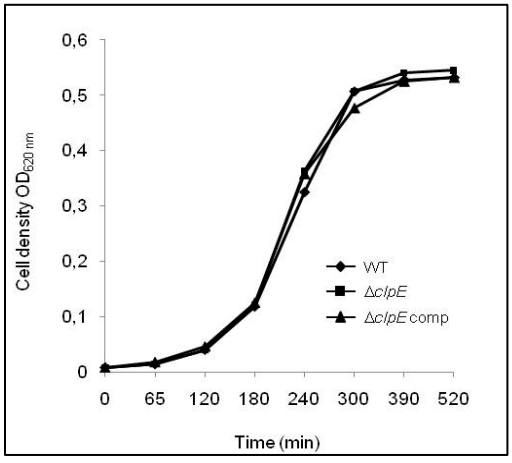


Fig. 23. Growth of *clpE*-deficient strain. Strains of wild-type (WT), Δ*clpE* and Δ*clpE* complemented with *clpE in trans* were cultivated in THY at 37°C, 5% CO2. Culture density was measured regularly at $OD_{620\ nnm}$. The experiment was performed three times independently. A representative result is shown.

4.2. Biofilm formation

The ability of Δ*clpE* mutant to form biofilm in rich medium on polystyrene plastic plates was monitored after 24 h. Whereas Δ*clpX* and Δ*clpP* formed more biofilm than the wild-type strain, Δ*clpE* produced twice less biofilm compared to the parental or the complemented mutant (Fig. 24). Thus, ClpE is involved in the formation of *S. pyogenes* biofilm, however its role is reverse to that of ClpP and ClpX.

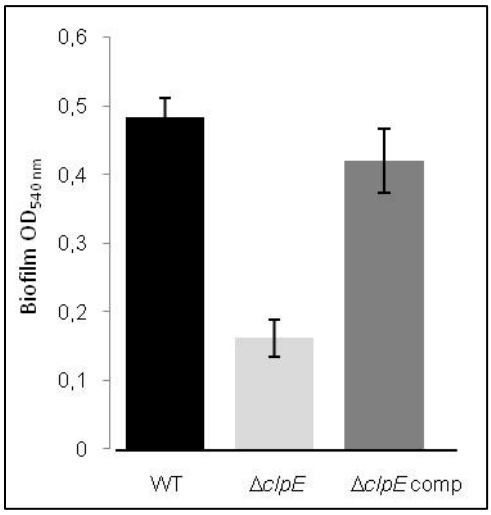


Fig. 24. ClpE is involved in biofilm formation. Strains of wild-type (WT), Δ*clpE* and Δ*clpE* complemented with *clpE in trans* were cultivated in triplicates for 24 h in C-medium at 37°C, 5% CO2. After washing with PBS, attached cells were dyed with 0.2% crystal violet. Cells were then retrieved with 1% SDS solution and cell density was determined at $OD_{540\ nm}$. The values shown are the average of three inocula ± SD. A representative result is shown.

4.3. Aggregation

As biofilm formation linked to cell to cell aggregation, the sedimentation rate of wild-type, Δ*clpE* and Δ*clpE* complemented strain was examined. Δ*clpE* aggregated with slightly slower rate compared to the wild-type (Fig. 25).

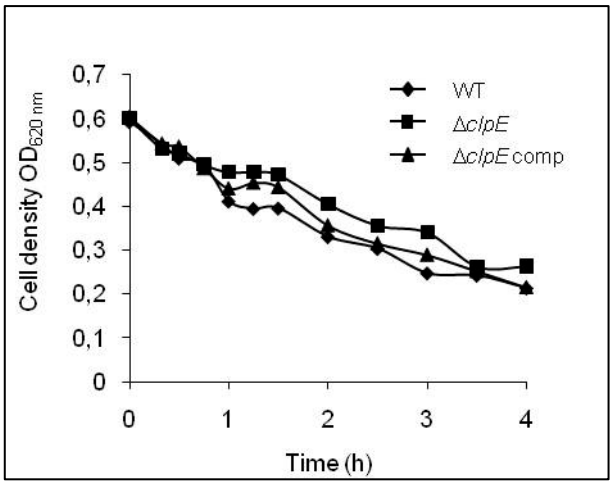


Fig. 25. Deletion of *clpE* affect streptococcal aggregation. Strains of wild-type (WT), Δ*clpE* and Δ*clpE* complemented with *clpE in trans* were cultivated over-night in THY medium at 37°C with shaking. Cultures were kept standing. Cell density of the upper phase of culture was determined at $OD_{620\ nm}$. The experiment was performed three times independently. A representative result is shown.

4.4. Survival under critical conditions

4.4.1. Lethal temperature

In other Gram-positive bacteria, the main role of the ClpATPase ClpE was to respond to heat-shock [82, 104]. Here, tolerance to lethal temperature was assessed by exposure of the wild-type, the Δ*clpE* mutant and the complemented mutant at 44°C. After an hour at this temperature, the wild-type surprisingly grew reaching the double amount of cells. This could be explained by the fact that the volume of culture was bigger than for the experiment using Δ*clpX* and Δ*clpP* therefore a longer time was needed for the media to acquire the desired temperature. However, after 2 h of heat-shock, 80% of the wild-type strain survived compared to only 50% of the Δ*clpE* strain (Fig. 26). A similar ratio was observed after 3 h. Thus, ClpE plays a positive role in the resistance to lethal temperature.

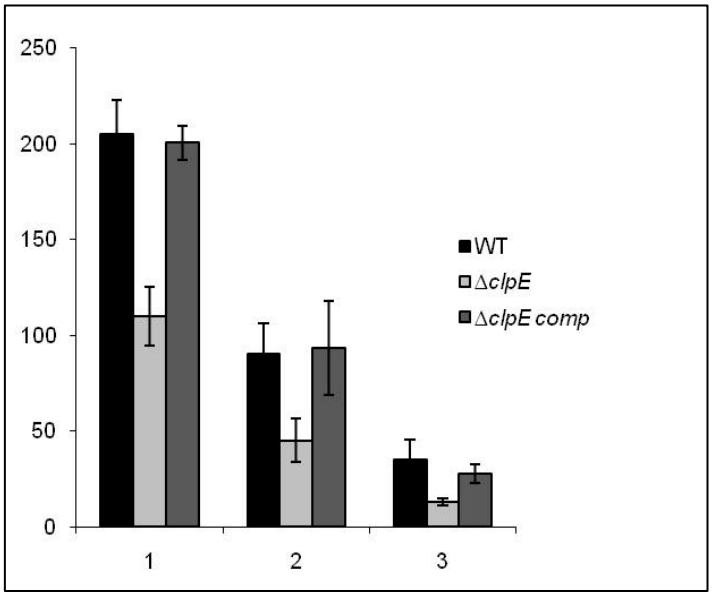


Fig. 26. ClpE is involved in the resistance to lethal temperature. Strains of wild-type (WT), Δ*clpE* and Δ*clpE* complemented with *clpE in trans* were cultivated in THY at 37°C, 5% CO_2. At mid-log phase, culture temperature was shifted to 44°C. Aliquots were collected every hour and colony forming units (cfu) / ml were determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. A representative result is shown.

4.4.2. Oxidative stress

Knowing that Δ*clpX and* Δ*clpP* were more sensitive to oxidative stress, the survival of Δ*clpE* after H_2O_2 exposure was examined. As shown in Fig. 27, no significant differences in survival between wild-type strain and Δ *clpE* mutant were observed. Consequently, it appears that ClpX and ClpP but not ClpE are involved in the response to oxidative stress.

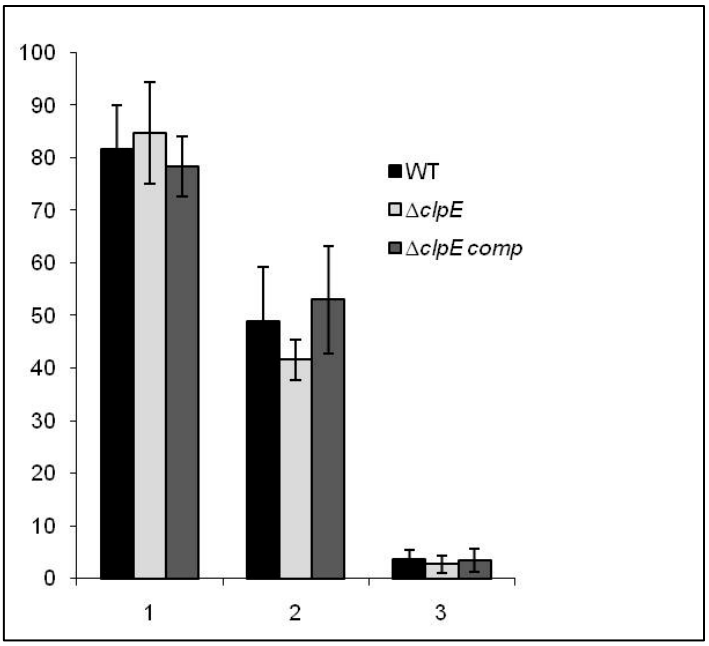


Fig. 27. **ClpE is not involved in the response to oxidative stress**. Strains of wild-type (WT), Δ*clpE* and Δ*clpE* complemented with *clpE in trans* were cultivated in THY medium at 37°C, 5% CO_2. At mid-log phase, 4 mM H_2O_2 were added to the culture. Aliquots were collected every hour and the ratio of colony forming unit (cfu) / ml was determined by plating dilution series onto TSA blood in triplicates. The values shown are mean survival percentages ± SD. A representative result is shown.

4.5. Exoprotein expression

To assess the role of ClpE in the regulation of virulence, the exoprotein expression pattern of the wild type and Δ*clpE* strains were compared by SDS-PAGE. No obvious differences were observed between the respective exoprotein patterns (data not shown).

To confirm the absence of differences in exoprotein expression between wild-type and Δ*clpE* strain, the expression of some key virulence factors: SpeB, M protein, Sic and Ska, was studied by western blot analysis. Interestingly, some differences were observed. Ska was more expressed in the *clpE*-deficient strain compared to the wild-type but this increase of expression was lower than the one observed in Δ*clpX* and Δ*clpP* strains. In the case of M protein, its expression was increased at comparable level in the three Δ*clp*-deficient strains compared to the wild-type (Fig. 28). However, no differences were observed between Δ*clpE* and wild-type for the expression of SpeB and Sic. In conclusion, ClpE is involved in the regulation of expression of Ska and M protein but not of SpeB and Sic.

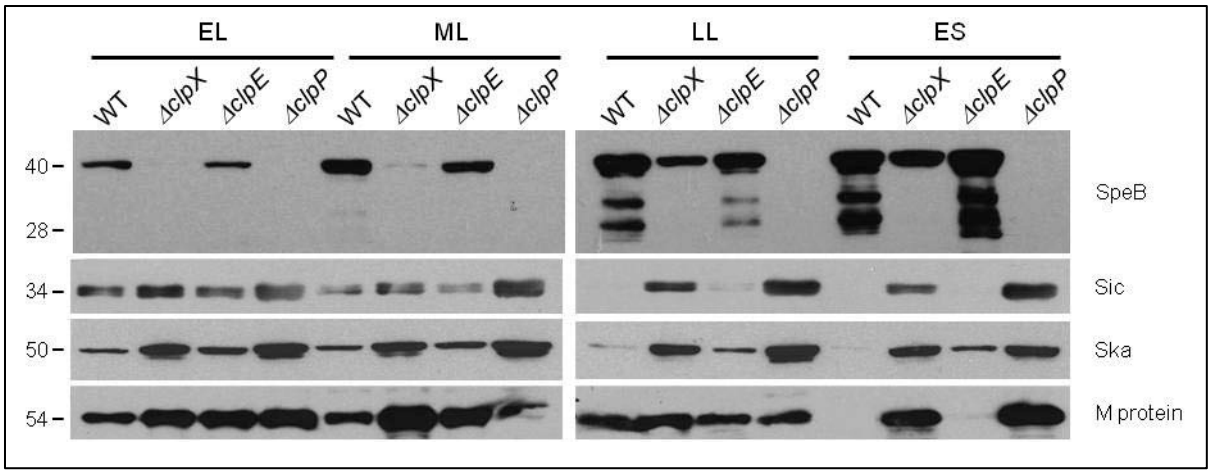


Fig. 28. Virulence factor expression. Western blot analysis of wild-type, Δ*clpX*, Δ*clpE* and Δ*clpP* exoproteins harvested during growth: EL (early-log), ML (mid-log), LL (late-log) and ES (early-stationary). 5 µg of exoproteins were loaded on a 10% SDS-PAGE. Additionally, equal loading was determined by ponceau staining of the membrane. Proteins were detected with specific antibodies against SpeB, Ska, Sic and M protein. Sizes are indicated in kDa.

Discussion

Proteolysis is required in the cell for the natural turn-over of cytosolic proteins as well as in the regulation of metabolic pathways. Various studies have also shown that proteolysis plays an important role in the regulation of virulence. In Gram-positive bacteria, Clp proteolytic complexes are involved in pathogenicity by degrading misfolded proteins accumulating upon stress conditions that occurr during infection. They also regulate the expression of key virulence factors by interfering with their regulators [99, 101, 109, 114, 121]. The goal of our study was to determine the role of Clp-mediated proteolysis in the human pathogen *S. pyogenes,* agent of pharyngitis and life threatening diseases such as flesh eating disease and toxic shock syndrome. To assess this question, we performed in-frame deletion mutants of each *clp* gene involved in proteolysis, the Clp ATPase encoding genes *clpX, clpE* and *clpC* and the protease encoding gene *clpP*. Following extensive phenotypic analysis we were able to show that in *S. pyogenes,* Clp-mediated proteolysis is involved in cell morphology, biofilm formation, response to various stresses encountered during infection such as heat-shock, oxidative stress and osmotic stress, resistance to penicillin and adhesion to epithelial cells. Moreover, we showed that Clp proteins are involved in the transcriptional regulation of key streptococcal virulence factors interfering with the expression of the transcriptional regulator RopB, the regulator *fasX* RNA and regulators CovR and FasA.

1. *Clp* gene organization

In order to perform in-frame *clp* gene deletion in *S. pyogenes*, we analyzed the genetic organization of the *clpX clpE, clpC* and *clpP* loci in this pathogen's genome, predicting the promoter region, the terminator and various transcriptional regulator binding motifs. We could predict the presence of two distinct promoters P1 and P2 located upstream of *clpP,* respectively at -921 nt and -119 nt from the start of translation, suggesting that *clpP* can be transcribed as a mono-cistronic transcript as well as a bi-cistronic transcript including *upp,* a gene coding for an uracil phosphoribosyltransferase, located downstream of *clpP* (Fig. 4B). This genetic organization is similar to that observed in *S. mutans* [324]. Northern blot analyses confirmed that *clpP* is constitutively expressed as both mono- and bi-cistronic transcripts, however, an increase of mono-cistronic expression during late-exponential phase was observed (Fig. 6). Interestingly, during heat-shock, we observed an induction of expression of *clpP* after 15 min from the promoter P2 suggesting that the regulation of *clpP*

expression occurs at the level of the mono-cistronic transcript (Fig. 7). Upstream of the promoter P2, we located a CtsR-binding site consistent with the fact that in other Gram-positive organisms, *clpP* is regulated by the transcriptional repressor CtsR. However, no CtsR binding sites were identified upstream of the promoter P1 (Fig. 4B). Together our data indicate that the induced expression of the *clpP* mono-cistronic transcript observed upon heat-shock or at the entry to stationary phase is regulated by CtsR. Thus, *clpP* but not *upp* is part of the CtsR regulon. In addition, the presence of a weak transcriptional terminator downstream of *upp* suggests that *upp* can as well be expressed on its own. Nevertheless, it is surprising to see, that co-transcription of *clpP* with *upp,* encoding an uracil phosphoribosyl transferase, an enzyme involved in *S. mutans* in the microbial pyrimidine salvage pathway, is observed in all streptococci. This enzyme catalyzes the conversion of uracil and 5-phosphoribosyl-α-1-pyrophosphate to UMP and pyrophosphate. Co-transcription of *clpP* and *upp* suggests a link between ClpP-mediated proteolysis and nucleotide biosynthesis pathway which might be important during infection where nucleotide starvation can occur. Further experiments are required to determine the link between ClpP proteolysis and *upp*.

In the case of *clpX*, our predictions led to the identification of one promoter region upstream of *clpX* and a weak terminator downstream of the *clpX* transcript (Fig. 4C). However, the ribosome binding site of *yihA*, gene coding for a GTP binding protein orthologue of *engB* or *ysxC*, located downstream of *clpX* is overlapping the 3'end of the *clpX* transcript, suggesting that these two genes are expressed in an operon. Northern blot analysis revealed the presence of 2 distinct transcripts matching with the 2 different terminators of transcription: downstream of *clpX* and downstream of *yihA* indicating that *clpX* is expressed both as mono- and bi-cistronic transcripts (Fig. 7). The co-organization of *clpX* and *yihA* is conserved in *S. aureus, S. pneumoniae, S. mutans* and *S. pyogenes* and it was already shown that *clpX* and *ysxC* are expressed in an operon in *S. aureus* (Fig. 5)[83]. YihA or YsxB, which have been reported to be essential in *B. subtilis, E. coli, S. pneumoniae* and *S. aureus,* are involved in bacterial growth and cell division. It is required for the biogenesis of the large ribosomal subunit 50S [320-321, 327-329]. Noteworthly, our efforts to generate a *yihA* deficient strain in *S. pyogenes* remained unsuccessful most likely indicating that YihA is also essential in *S. pyogenes.* Interestingly in *E. coli,* YihA is expressed in an operon with Lon, another cytosolic protease related to Clp proteins [330]. This would suggest that proteolysis is involved in YihA regulation and that ClpX mediates this function in *S. pyogenes*.

2. Heat regulation of *clp* genes

Our results show that upon exposure to high temperature, *clpP* was overexpressed already after 15 min whereas an increased expression of *clpX* was only observed after 30 min. Consequently, *clpX* cannot be characterized as a classical heat-shock gene due to the delayed response upon heat-shock. Moreover, we indentified a CtsR-binding site in the promoter of *clpP, clpE* and *clpC* operon suggesting that in *S. pyogenes* as in all the Gram-positive studied so far, ClpP, ClpE, ClpC and CtsR expression is under the control of the negative transcriptional regulator CtsR. However, no CtsR-binding site was found in the vicinity of the *clpX* promoter consistent with the observation made in other Gram-positive organisms that ClpX is not part of the CtsR regulon and therefore its expression is not influenced by heat. In the model organism *B. subtilis*, CtsR has been shown to be regulated by ClpCP-mediated proteolysis with the help of the adaptor protein MscB, which gene is part of the *clpC* operon [96]. However in *S. pyogenes,* like in *S. pneumoniae* and *S. mutans*, the *clpC* operon is only composed of *ctsR* and *clpC* and neither *mscB* nor *mcsA* homologues could be identified in the genome. This indicates that in Streptococci, the regulation of CtsR is still probably involving the ClpCP complex, but with a different adaptor protein, which remains to be identified. Alternatively, CtsR could be regulated by a completely different mechanism.

3. Clp proteins are required for cell division

To study the role of Clp proteins in the pathogenicity of *S. pyogenes*, we engineered *clp*-deficient mutants in the M1 serotype clinical isolate, SF370, the complete genome of which is publicly avalaible. Interestingly, the *clpX*- and *clpP*-deficient strains exhibited a slight delay of growth of approximately 1 h and 2 h, respectively and they reached a lower cell density at stationary phase compared to the wild-type strain. This observation was already made for *clpP*-deficient strains in the Gram-positive bacteria *B. subtilis, L. monocytogenes, S. aureus, S. pneumonia* and *S. mutans* and for the *clpX*-deficient strain in *S. mutans*, where another protease, the Lon protease is missing [91, 99, 101, 105, 115, 123]. However, in Gram-negative bacteria such as *E. coli* and *S. typhimurium*, where the Lon protease is present, no *clp* mutants exhibited a delay in growth [331-332]. Thus, in the absence of Lon and Clp-mediated proteolysis, the accumulation of misfolded proteins and aggregates may be too significant for the cells to replicate at their native pace [102]. In our case, the amount of bacteria showed that the generation times of the mutants were not reduced, however they started replicating 1 h

after transferring the cells to the new medium. This prolonged lag phase could be explained by a delayed adaptation to the new medium after inoculation. Moreover, at stationary phase we observed a lower density of cells but the bacterial count was similar comparing mutant and wild-type stains, thus suggesting a reduction in cell size of the mutants. In fact, electron microscopy studies of *clpP* and *clpX* mutant strains revealed that both strains exhibited smaller cells than the wild-type (Fig. 12). This result is consistent with the finding that in *S. aureus*, the *clpP*-deficient strain is 0.8 times smaller than the wild-type [115]. Furthermore, microscopic studies revealed that the streptococcal chain morphology was affected in the mutants. Indeed, the *clpP* mutant chains showed a defect in cell division whereas the *clpX* mutant chains were longer compared to the wild-type with an increased aggregation of these chains. In the case of the *clpX* mutant, this phenotype is consistent with phenotypes observed in *B. subtilis clpP* mutant and *S. pneumoniae clpC* mutant, which exhibit longer chains due to a defect in cell dissociation explained by a decreased level of autolysin [97, 103, 121]. Morever, *clpX*- and *clpP*-deficient strains displayed a reduced expression of *ropB*, which positively regulates autolysin expression [273]. Therefore, ClpX and ClpP may also be involved in the activation of autolysin in *S. pyogenes*. The case of ClpP is more complicated as the *clpP* mutant cells appeared to be affected in cell division. The first hypothesis will be that due to an abnormal accumulation of misfolded proteins and aggregates, cells are not fit to divide. However, studies showed that Clp-mediated proteolysis is directly involved in the regulation of cell cycle. For example, in *E. coli*, the ClpXP complex is responsible for the degradation of the cytosqueletal protein FtsZ promoting the septum formation and in *L. monocytogenes*, ClpC and ClpE are required for the septum formation [104, 333]. A recent study performed in our lab revealed that in *L. monocytogenes*, the ClpCP complex degrades *in vivo* the chromosome segregating factor ParB (Spiess, 2010 in press). Moreover, *clpX* is expressed in an operon with YihA, a protein required for cell division [320]. Our results bring striking evidences that in *S. pyogenes*, ClpP-mediated proteolysis is required for the regulation of the cell cycle but the role of each ATPase in this process remains unclear.

4. Clp proteolysis regulates cell to cell aggregation

Our results show that the ClpXP complex inhibits the formation of biofilms and reduces cell to cell aggregation. On the other hand, the chaperone ClpE promotes biofilm formation and cell aggregation which is consistent with findings obtained in *S. mutans* and indicates a

conserved role for Clp proteins in aggregation and biofilm formation in Streptococci [91, 105]. However, we believe that the variations in biofilm formation in the mutants are due to differences in cell to cell aggregation, therefore the role of ClpX, ClpP and ClpE on biofilm formation may only be indirect. The fact that the *clpP*- and *clpX*-deficient strains exhibit a defect in cell division and chain formation could partly explain the increase in bacterial aggregation, though other factors involved in adhesion could be assumed to participate in these phenotypes as well. Only recently, *S. pyogenes* was found to produce pili-like structures at its surface, involved in cell to cell aggregation, biofilm formation and adhesion to epithelial cells [154, 197]. Chaperone proteins such as GroEL participate in the pili assembly therefore the chaperone activity of ClpE may be involved in the pilus assembly in *S. pyogenes* [334]. On the other hand, ClpXP mediated degradation would inhibit pili formation. The second interesting factor is the hyaluronic acid capsule involved in cell to cell interaction and in the binding to epithelial cells. In *S. pneumoniae*, an increased expression in the capsule genes in the *clpP* mutant was observed [87]. In *S. pyogenes*, the expression of the *has* operon, responsible for the hyaluronic acid synthesis, is repressed at the transcriptional level by CovR, the effector of the two-component system CovR/CovS. In our *clpP*- and *clpX*-deficient strains, the expression of CovR is visibly reduced, suggesting an augmentation of hyaluronic acid production [181, 183-186]. A thickening of the capsule could explain the increase of cell aggregation, the increase in interaction with epithelial cells, the increased resistance to penicilin and the defect in transformation observed in the *clpX* and *clpP* mutants. Consequently, in *S. pyogenes* the ClpXP complex would activate the expression of CovRS leading to the repression of capsule expression (Fig. 29).

5. Clp proteolysis and stress response

Stress conditions lead to an increase of misfolded proteins and aggregates, which need to be proteolyzed in order for the bacteria to survive the encountered stresses. Therefore proteolysis is required for survival upon stresses. In Gram-positive bacteria, Clp-mediated protolysis was shown to be involved in the response to heat-shock, suboptimal temperature exposure, oxidative, osmotic and acidic stress (Cf. Introduction Table 3). Here, our data show that ClpP and ClpX are required to survive oxidative stress in *S. pyogenes*. The fact that the *clpE*-deficient strain showed no difference in survival upon H_2O_2 treatment compared to the wild-type emphasizes the observation that ClpX in association with ClpP are required to

survive oxidative stress in *S. pyogenes*. Interestingly, Spx, a transcriptional regulator of oxidative response genes, was found to be a substrate of the ClpXP machinery in *B. subtilis* and *S. mutans* [55, 71, 105]. Two homologues of Spx were identified in the genome of *S. pyogenes*, Spy_1249 and Spy_2115, indicating that they could be potential substrates of the ClpXP complex linking ClpXP with the response to oxidative stress. In *S. aureus*, the resistance to oxidative stress and osmotic stress is carried out by the ClpXP complex [83]. However, in the case of *S. pyogenes*, ClpP but not ClpX appears to be involved in the response to high salt concentration exposure. Therefore we can presume that in *S. pyogenes*, another ATPase than ClpX is associated with ClpP as previously observed in *L. monocytogenes*, where ClpC and ClpP are required for the response to osmotic stress [99, 104, 111]. Our phenotypic analyses reveal that the *clpP* and *clpX* mutants are more resistant to lethal temperature than the wild-type strain. In the case of ClpX, this phenotype was already observed in *S. aureus* [83, 101]. As previously mentioned, ClpX is not part of the CtsR regulon and its expression is not regulated by heat therefore it is not surprising that ClpX is not involved in the heat-shock response in *S. pyogenes*. However, ClpP of Gram-positive bacteria is known to be regulated by CtsR and its expression is activated after heat stress. Moreover, in other Gram-positive organisms including *L. monocytogenes*, *S. aureus*, *S. pneumoniae* and *S. mutans*, ClpP was systematically shown to be involved in heat-shock response. Therefore, our result is not consistent with the previously reported requirement of ClpP during heat-shock in other Gram-positive bacteria. However, the reduced expression of the transcriptional regulator RopB in the *clpP* mutant could be an explanation. Indeed, the streptococcal *ropB*-deficient strain was associated with an increase in resistance to high temperature and an activation of *clpE* expression [272]. ClpE, which is tightly regulated by CtsR, is required for resistance to heat-shock in *L. monocytogenes* and *S. pneumoniae*. Our experiments show that ClpE is also required for *S. pyogenes* to resist to lethal temperature [88, 104]. Therefore, the increased tolerance to heat of the *clpP*-deficient strain could be explained by a decreased expression of RopB leading to an overexpression of ClpE. Finally, we observed that *clpX*- and *clpP*-deficient strains are more resistant to penicillin than the wild-type. As previously mentioned, the putative increase in capsule production in the mutants could explain the increased resistance to penicillin of the mutants. Furthermore, RopB contributes to the penicillin mediated killing in *S. pyogenes*, suggesting that a reduced expression of *ropB* observed in the mutants could confer an increased resistance to penicillin [275].

6. Clp involved in regulation of virulence

One of the aims of our study was to determine the impact of the Clp-mediated proteolysis on the virulence of *S. pyogenes*. The observation that *clpX* and *clpP* mutants display an increase in cell aggregation led us to hypothesize that ClpX and ClpP were involved in the regulation of adherence of *S. pyogenes*. Adhesion assays to laryngeal epithelial cells showed that the *clpX* and *clpP* mutants adhere better to epithelial cells compared to the wild-type. This could be explained by an increase in cell to cell aggregation observed in the mutants and the putative thickening of the capsule. However, we cannot exclude that an overexpression of other adhesins or fibronectin-binding proteins is responsible for the increased streptococcal adherence in the *clp* mutants. To determine the impact of ClpE, ClpX and ClpP on virulence factor expression, we compared the exoprotein expression profile of the three *clp*-deficient strains with the wild-type. Mass spectrometry analyses confirmed by Western blot revealed an overexpression of M protein, Ska, Sic and a repression of SpeB expression in the *clpX*- and *clpP*-deficient mutants compared to the wild-type (Fig. 18-19). In the case of M protein and Ska, we observed a constant increase of expression throughout the growth of the mutants. This indicates that ClpX and ClpP negatively regulate the expression of M protein and Ska. The expression of Sic in the mutants was slightly different as in late-exponential and stationary phase, Sic amounts faded away in the wild-type but remained equal in the mutants. At the RNA level, *sic* was overexpressed in both mutants during exponential phase but not at early-stationary phase indicating an enhanced protein stability of Sic in the mutants during stationary phase. Consequently, Sic could be a substrate for the ClpXP proteolytic complex explaining its increased stability in the mutants. The expression of SpeB in the *clpX* and *clpP* mutants was reduced indicating that ClpX and ClpP positively regulate SpeB expression. However we observed a discrepancy in phenotypes comparing the two mutant strains. In fact, in the *clpP* mutant the expression of SpeB was totally abolished whereas in the *clpX*-deficient strain, the expression of SpeB was delayed. In addition, while SpeB was cleaved into its mature form in the wild-type, the *clpX* mutant exhibited only the zymogen form of 40 kDa suggesting a defect in SpeB expression and maturation. The maturation of SpeB involves SpeB itself and the cell wall-associated protease HtrA [196, 223]. Therefore, ClpX and ClpP may be involved in the proper folding of SpeB and/or in the regulation of HtrA expression. The weaker phenotype observed in Δ*clpX* suggests that ClpP activates SpeB expression in association with ClpX but as well with another ATPase. The absence of phenotype

concerning SpeB production in the *clpE* deficient strain suggests that ClpC might be the other Clp ATPase that plays a role in this regulation.

After concluding that ClpX and ClpP are involved in the regulation of major streptococcal virulence factors expression we were interested in determining the level of regulation. Northern blot analysis revealed that ClpX and ClpP act already at the transcriptional level as the RNA level is consistent with the protein level observed in Western blot (Fig. 20). We observed an increased expression of the M protein encoding gene, *emm*, *sic* and *ska* and a decreased expression of *speB* as well as the streptolysin encoding genes, *sls* and *slo* and the superantigen encoding gene *speC*. In the *clpP* mutant, the expression of *speB* was totally abolished revealing that ClpP-mediated proteolysis is required for *speB* expression. We can conclude that ClpX and ClpP are involved in the inhibition of *emm, ska* and *sic* expression and the activation of *speB, sls, slo* and *spec* expression. Similarly to the activation of streptolysin expression, ClpP was shown to participate in the activation of the listeriolysin O encoding gene, *llo* in *L. monocytogenes* suggesting that ClpP contributes to the escape of the host immune response in both pathogens [99]. Interestingly, M protein, Ska and Sic are virulence factors involved in the colonization of the host therefore expressed early during infection whereas SpeB, SpeC and Streptolysins S and O are required for the spreading into the organism expressed later during the infection [236]. This suggests a growth phase-dependent regulation of virulence factor expression by the ClpXP proteolytic complex. In *E. coli*, the ClpXP complex is involved in the regulation of the sigma factor σ^s and in *B. subtilis*, ClpX degrades RsiW the negative regulator of the sigma factor σ^w [48, 50]. In *S. aureus*, the coordination of growth phase-dependent expression is mediated by the *agr* locus and the ClpXP complex was shown to activate *agr* expression [119]. In the absence of Sigma factors in *S. pyogenes*, the growth phase-dependent gene regulation is carried out by stand-alone transcriptional regulators such as Mga, RofA and RopB, which respond to environmental signals [236]. Therefore we can speculate that in *S. pyogenes*, the ClpXP proteolytic complex influences these regulators. In the absence of specific antibodies directed against Mga, RofA, Nra and RopB, we analyzed the RNA level of these regulators in the wild-type, *clpX- and clpP-deficient* strains. No differences were observed for *mga, rofA* and *nra* while the expression of *ropB* was decreased in the mutants. Consequently, ClpX and ClpP interfere with RopB expression known to be the regulator of the entry into stationary phase [236].

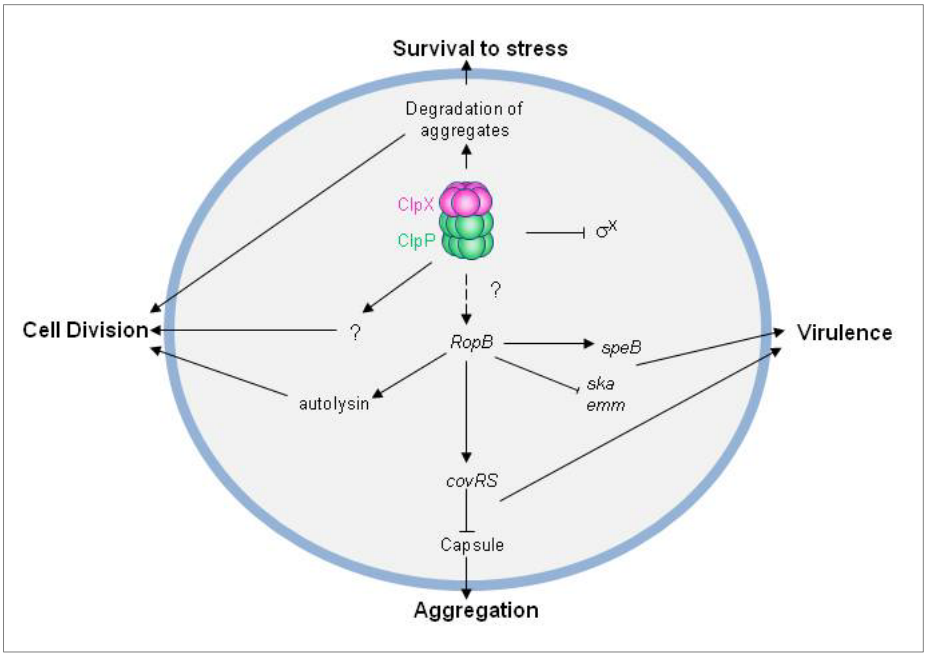


Fig. 29. Model of the impact of ClpXP-mediated proteolysis in *S. pyogenes.*

RopB is the main activator of SpeB transcription, which would explain the reduction and absence of SpeB expression in the *clpX* and *clpP* mutants, respectively [265, 269]. In addition, RopB represses genes necessary for colonization by inhibiting the expression of *mga* and its regulon in the entry of stationary phase [239]. *emm*, *sic*, and *ska* are part of the *mga* regulon, thus their increased expression in the mutants could be explained by the decrease in *ropB* expression. However, no striking differences in *mga* expression were observed in the mutants. It is possible that the increased expression of *mga* was not visible in our Northern blot or that RopB acts at the translational level. To summarize, the *clpP* and *clpX* mutants exhibit an increased expression of colonization factors and a decreased expression of dissemination factors. Therefore, we could envisage that during infection, both mutants will colonize the host, faster and more efficiently than the wild-type however they may not managed to spread in the host which at the the end, will make them less virulent than the wild-type. In addition to stand-alone regulators, we examined the expression of other regulators such as PerR, MalR, CodY and two-component systems effectors and regulators. We observed a reduction of transcription of *fasA* and *covR*, both encoding effectors of the two-component systems FasBCA and CovRS, respectively and *fasX* a small regulatory RNA activating the expression of the FasBCA operon (Fig. 22). Notably, RopB activates the expression of CovR and the Fas operon. Therefore, most of the phenotypes we observed in the *clpP* and *clpX* mutants during our study can be associated with the decrease in *ropB*

expression. RopB regulates autolysin involved in cell segregation, capsule expression involved in aggregation and adherence, penicillin resitance and virulence factor expression by repressing *mga* and activating CovRS and FasBCA. In *S. aureus*, the growth phase-dependent gene regulation is mediated by the *agr* locus composed of RNAII and RNAIII, which are divergently transcribed. RNAII encodes a two-component system, which activates the expression of RNAIII, which is an activator of virulence factor expression [335]. The ClpXP complex was shown to activate transcription of the *agr* locus [119]. We can conclude that the main role of the ClpX and ClpP proteins in *S. aureus* as well as in *S. pyogenes* is to promote the entry into stationary phase via regulation of *agr* and *ropB*, respectively (Fig. 29). The simplest hypothesis would be that the ClpXP complex degrades *in vivo* an inhibitor of RopB expression, however, no factor involved in its expression has been identified so far. Finally, RopB is expressed from two distinct promoters located in the intergenic region between *speB* and *ropB* where *speB* is transcribed in the opposite direction of *ropB* (Fig. 21). In the wild-type, at early stages of growth, *ropB* is expressed from P1 and in the late-exponential/early-stationary phase, its expression switches to the second promoter. However, in the *clpX* and *clpP* mutants we observed an increased transcription from the promoter P1 but never from the second promoter P2. Previous studies of this region concluded that SpeB is expressed from two disctinct promoters Pa and Pb and that RopB binds in the region of the proximal promoter of SpeB corresponding to the promoter P1 of *ropB*. We could hypothesize that a factor is responsible for the switch of *ropB* promoter activity promoting the entry into stationary phase and that the ClpXP complex could interfere at this level. Thus, the link between the Clp-mediated proteolysis and RopB remains unknown and deserves to be investigated in the future.

7. Conclusion

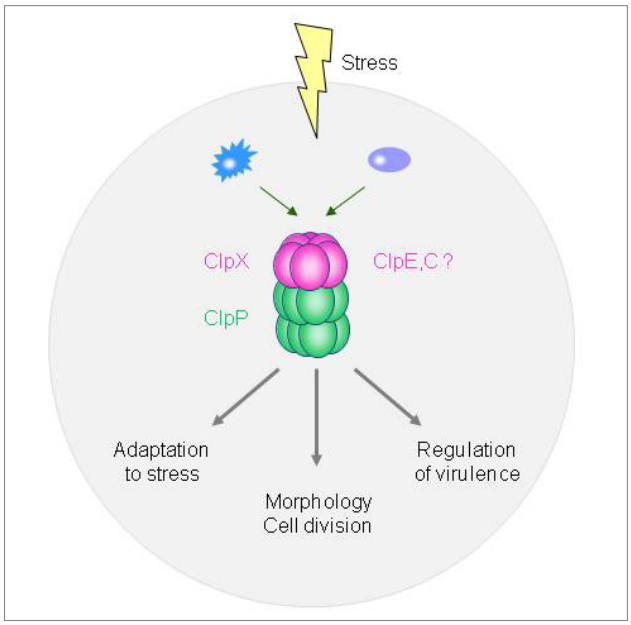


Fig. 30. Summary of the functions of the ClpXP proteolytic complex in *S. pyogenes.*

Our study shows that in *S. pyogenes*, similar to other Gram-positive pathogens, Clp-mediated proteolysis is required for the survival under various stresses and adaptation to new environmental conditions encountered during infection. Moreover, we showed that Clp proteolysis is essential for cell separation during cell division. Furthermore, Clp proteins respress the expression of virulence factors required for colonization (*emm, ska, sic* among others) and activates the expression virulence factors necessary for the spreading and persistence (such as *speB, slo, sls*) by activating the expression of their main transcriptional regulator, RopB (Fig. 30). The main challenge in the future will be to identify by which mechanisms the ClpXP complex activates RopB expression. In parallel, it will be necessary to study in more detail, the other Clp proteins associated with proteolysis, ClpC and ClpE, to determine their functions in *S. pyogenes*. Another aspect of research would be to elucidate the interactions and mutual modulations within the Clp network. The fact that ClpP is a direct target of ADEPs [137], a new class of antibiotics, stresses the importance of understanding how the Clp-mediated proteolysis interferes with virulence, to further develop new drugs directed especially against multi-resitant Gram-positive pathogens.

Appendix

Table 1: Bacterial strains used in the Clp's study

Code	Relevant characteristics	Source
S. pyogenes		
EC904	SF370 M1 serotype wild-type	ATCC 700294
ClpX		
EC1463	EC904(pEC272)replicating Clone 3	This study
EC1468	EC904::pEC272 integrated clone 3.I	This study
EC1469	EC904::pEC272 integrated clone 3.II	This study
EC1470	EC904::pEC272 integrated clone 3.III	This study
EC1488	EC904 Δ*clpX* clone 3.I.22	This study
EC1489	EC904 *clpX* WT clone 3.I.23	This study
EC1490	EC904 *clpX* WT clone 3.I.24	This study
EC1491	EC904 *clpX* WT clone 3.I.25	This study
EC1492	EC904 *clpX* WT clone 3.I.44	This study
Spy_0886		
EC1464	EC904 (pEC271) replicating clone 3	This study
EC1465	EC904::pEC271 integrated clone 3.I	This study
EC1466	EC904::pEC271 integrated clone 3.II	This study
EC1467	EC904::pEC271 integrated clone 3.III	This study
EC1493	EC904 *Spy_0886* WT clone 3.II.17	This study
EC1494	EC904 *Spy_0886* WT clone 3.II.25	This study
EC1495	EC904 *Spy_0886* WT clone 3.II.30	This study
EC1496	EC904 *Spy_0886* WT clone 3.II.38	This study
EC1497	EC904 *Spy_0886* WT clone 3.II.1	This study
EC1498	EC904 *Spy_0886* WT clone 3.II.18	This study
EC1499	EC904 *Spy_0886* WT clone 3.II.27	This study
No Spy_0886 clones could be obtained.		
ClpP		
EC1379	EC904 (pEC217) replicating clone A	This study
EC1380	EC904 (pEC217) replicating clone B	This study
EC1381	EC904 (pEC217) replicating clone C	This study
EC1382	EC904::pEC217 integrated clone A.1	This study
EC1383	EC904::pEC217 integrated clone A.2	This study
EC1384	EC904::pEC217 integrated clone A.3	This study
EC1520	EC904 ΔclpP clone A.1.12	This study
EC1521	EC904 ΔclpP clone A.1.18	This study
EC1522	EC904 ΔclpP clone A.1.19	This study
EC1523	EC904 clpP WT clone A.1.1	This study
EC1524	EC904 clpP WT clone A.1.2	This study

ClpE		
EC1427	EC904 (pEC266) replicating clone A	This study
EC1428	EC904 (pEC266) replicating clone B	This study
EC1429	EC904::pEC266 integrated clone B.1	This study
EC1430	EC904::pEC266 integrated clone B.2	This study
EC1431	EC904::pEC266 integrated clone B.3	This study
EC1435	EC904 Δ*clpE* clone B.1.8	This study
EC1445	EC904 Δ*clpE* clone B.1.2	This study
EC1446	EC904 Δ*clpE* clone B.1.9	This study
EC1447	EC904 *clpE* WT clone B.1.1	This study
EC1450	EC1446 (pEC85)	This study
EC1451	EC1446 (pEC259)	This study

ClpC		
EC1510	EC904 (pEC275) replicating clone B	This study
EC1511	EC904 (pEC275) replicating clone C	This study
EC1512	EC904 (pEC275) replicating clone D	This study
EC1513	EC904 (pEC275) replicating clone E	This study
EC1514	EC904::pEC275 integrated clone D.1	This study
EC1515	EC904::pEC275 integrated clone D.2	This study
EC1516	EC904::pEC275 integrated clone D.3	This study
EC1535	EC904::pEC275 integrated clone C.1	This study
EC1536	EC904::pEC275 integrated clone C.2	This study
EC1537	EC904::pEC275 integrated clone C.3	This study
EC1550	EC904 Δ*clpC* clone C.1.4	This study
EC1551	EC904 Δ*clpC* clone C.1.5	This study
EC1552	EC904 *clpC* WT clone C.1.10	This study
EC1553	EC904 *clpC* WT clone C.1.6	This study

E. coli

RDN204	Top10 - Host for cloning	Lab strains
EC480	TOP10 (pEC84)	Lab strains
EC1037	TOP10 (pEC85)	Lab strains
EC1046	TOP10 (pMAD)	Gift from Debarbouille
EC1434	TOP10 (pEC214)	This study

ClpX		
EC1460	TOP10 (pEC272) clone 3	This study
EC1461	TOP10 (pEC272) clone 4	This study
EC1462	TOP10 (pEC272) clone 7	This study

Spy_0886		
EC1457	TOP10 (pEC271) clone 3	This study
EC1458	TOP10 (pEC271) clone 5	This study
EC1459	TOP10 (pEC271) clone 9	This study

ClpP		
EC1147	TOP10 (pEC206)	This study
EC1157	TOP10 (pEC209) clone 5	This study
EC1158	TOP10 (pEC209) clone 7	This study
EC1363	TOP10 (pEC217)	This study
EC1509	TOP10 (pEC260)	
ClpE		
EC1366	TOP10 (pEC252)	This study
EC1367	TOP10 (pEC252) clone15	This study
EC1368	TOP10 (pEC254) clone14	This study
EC1422	TOP10 (pEC265) clone 2	This study
EC1423	TOP10 (pEC265) clone e	This study
EC1424	TOP10 (pEC266)	This study
EC1444	TOP10 (pEC259)	This study
ClpC		
EC1365	TOP10 (pEC253)	This study
EC1432	TOP10 (pEC267)	This study
EC1508	TOP10 (pEC275)	This study
EC2021	TOP10 (pEC401) clone 3	This study
EC2022	TOP10 (pEC401) clone 6	This study

Table 2: Plasmids used in the Clp's study

Plasmid	Relevant characteristics	Source
pEC81	*repAts*-pWV01, *ermAM/B*, ColE1	Lab construct
pEC84	*repAts*-pWV01, pJH1-*aphIII*, ColE1	Lab construct
pEC85	*repDEG*-pAMß1, pJH1-*aphIII*, ColE1	Lab construct
pEC214	pEC84ΩP*clpB*-ß-gal	This study
ClpX		
pEC230	pEC214Ω*clpX*KOdw	This study
pEC231	pEC230Ω*clpX*KOup	This study
pEC261	pEC85Ω*PclpXTT*	This study
pEC272	pEC214Ω*clpX*KO2up-dw	This study
Spy_0886		
pEC271	pEC214Ω*Spy_0886*KOup-dw	This study
ClpP		
pEC206	pEC84Ω*clpP*KOdw	This study
pEC209	pEC206Ω*clpP*KOup	This study
pEC217	pEC209ΩßgaI	This study
pEC260	pEC85Ω*PclpPTT*	This study
ClpE		
pEC252	pEC254Ω*clpE*KOup	This study
pEC254	pUC19Ω*clpE*KOdw	This study
pEC259	pEC85ΩP*clpETT*	This study
pEC265	pEC84Ω*clpE*KOupdw	This study
pEC266	pEC265Ωβgal	This study
ClpC		
pEC253	pUC19Ω*clpC*KOdw	This study
pEC267	pUC19Ω*clpC*KOup-*aphIII*-*clpC*KOdw	This study
pEC275	pEC214ΩclpCKO2up-dw	This study
pEC401	pEC85ΩP$_2$*clpCTT*	This study

Table 3: Oligonucleotides used in the Clp study

Oligo	*F/ R	Sequence 5' - 3'	Characteristics
OLEC644	F	ATGCACCTCGAG*TTAGCATATTATGTTGCCAACTG*	Bgal from pMad
OLEC645	R	ATGCACCCGCGG*GTCTAGTTAATGTGTAACGTAAC*	
ClpX			
OLEC1153	F	ATGCAGCTGCAG*TAGGACTAATTTCTTTGATATTAAA*	ClpX up fragment
OLEC1154	R	*CTGTTCATTTTGTGGTAAAAGCCAGTAAGTCACAAAAGTGCGTATCACCAAGG*	
OLEC1155	F	*CCTTGGTGATACGCACTTTTGTGACTTACTGGCTTTTACCACAAAATGAACAG*	ClpX dw fragment
OLEC1156	R	ATGCAGGAATTC*CTATTACGATATCGAACACCAAAA*	
OLEC807	F	*GTATTCACGCCTTCTATG*	PCR, Seq
OLEC808	R	*GCCAAATTTTTGCGCCCC*	
OLEC809	F	*CTCAAGAATTGCTAAAACG*	
OLEC810	R	*CGTTCTTTACTACTCAAG*	
OLEC847	F	*TGACTGAAGCAGGGTATG*	SB probe
OLEC850	R	*GATCTCCTCGATGCCATC*	
OLEC867	R	*GGAAGTCTTTTGCAATTC*	
OLEC868	F	*TTAAGGAGATGAGTCATG*	
OLEC963	F	ACTGAGCTGCAG*TGAGGAGATGATAAGCGATGCAAC*	ClpX complementation
OLEC827	R	ACTGAGGAATTC*GTATTTAAGACTTGTTCTTCAGCCA*	
Spy_0886			
OLEC1157	F	ATGCAGCTGCAGGCAGATGCAACTTCTTTGACTGAAG	Spy_0886 up fragment
OLEC1158	R	TAGATAAATCAACACCTATCGCTATGAACGTCCAGCGAGGGCAATTTC	
OLEC1159	F	GAAATTGCCCTCGCTGGACGTTCATAGCGATAGGTGTTGATTTATCTA	Spy_0886 dw fragment
OLEC1160	R	ATGCAGGAATTCGAAGGACAACTAGTGATTAGGCAAAACG	
OLEC1161	F	TCAAAACCCGTTTTAGAG	PCR, Seq
OLEC1162	R	CTGTGTCATGACTCATC	
OLEC1163	F	TGTCTCGTTTACAGAAAG	
OLEC1164	R	GACGCTAAAGCTCATTTG	
OLEC1270	F	ATGTCCCTGGTTATGGC	Spy_0886 complementation
OLEC1271	R	GCCACGAGGAATCTTATC	
ClpP			
OLEC562	F	ATGCAGCTGCAG*AAAGGTAGCTGATAAAAAATCAGG*	ClpP up fragment
OLEC563	R	ATGCAGGGATCC*AATACATTCTCCAATCTACAAGGG*	
OLEC564	F	ATGCAGGGATCC*AGAGGATCTCTGCAAAAGTATTCTAG*	ClpP dw fragment
OLEC565	R	ATGCAGGAATTC*GTTGCTCTAAAACAGTCGTTTTCCCT*	
OLEC863	F	*ATGATTTGACCTTTATTG*	PCR, Seq
OLEC864	R	*AAAGGGATCTGAACTGAC*	
OLEC865	F	*CTTTAAACCGACAGGCCG*	
OLEC866	R	*TGTTTTAGGGTCCATAGC*	
OLEC877	F	*GCTCTTATTCCTTGACGC*	SB probe
OLEC878	R	*GATGTTCTGCTGCGATAG*	
OLEC879	R	*AGCTAATTTTTTACCAGC*	
OLEC880	F	*GGGTTAACAACCCCCTAG*	
OLEC1018	F	ATGCAGCGTCAG*GTCTAGTTAATGTGTAACGTAACA*	ClpP complementation
OLEC1004	R	CCACTAGGTCATCTCTTTTTTACTGCGATCCTTAATTATATTATAGTC	
OLEC1003	F	GACTATAATATAATTAAGGATCGCAGTAAAAAAGAGATGACCTAGTGG	
OLEC965	R	ACTGAGGAATCC*AGGGATCTGAACTGACGTTTTAAC*	

ClpE			
OLEC573	F	ATGCAGTCTAGA*GGAAGTAGTCTTGAAAAGTCCAAG*	ClpE up fragment
OLEC574	R	ATGCAGGGTACC*AATATACCTCCGAGGGTTTATCTA*	
OLEC575	F	ATGCAGGGTACC*CGTTAGCTAAGAGAGTTTCTATAAG*	ClpE up fragment
OLEC576	R	ATGCAGGAATTC*TCTTTTAAGAAAATTGCTGGCTTC*	
OLEC859	F	*AAAGCTAACGCTAAAGCG*	PCR, Seq
OLEC860	R	*ATTTTATTTACCTTTCC*	
OLEC861	F	*CATGGCATTTTGAGGACG*	
OLEC862	R	*TAAGAAGACAACCATC*	
OLEC873	F	*AGGCAAGCAAGTGATTCG*	SB probe
OLEC874	R	*AACAGAAGGTTCATCAAC*	
OLEC875	R	*ATCTTTATTGTAATGAAC*	
OLEC876	F	*TTACCCAAGCACCAAAAC*	
OLEC966	F	ACTGAGGGATCC*CCGCCATCTGTCACCATCAACTCC*	ClpE complementation
OLEC967	R	ATCGAGGAGCTC*TTCTTCATTATACTAAATTTTTGC*	
ClpC			
OLEC1262	F	ATGCACCGTCAG*TTTATGAGTGCCTGCAAGGACAAAAC*	ClpC up fragment
OLEC1263	R	ATGCACGAATTC*TTAAGCGGTCCATGCTAGCAGATTCG*	
OLEC1264	F	*CTTTTTCTGCCAATGTAGTAATAATAGCTACCATAGCTAACAAAACATG*	ClpC dw fragment
OLEC1265	R	CATGTTTTGTTAGCTATGGTAGCTATTATTACTACATTGGCAGAAAAAG	
OLEC855	F	*CAATAAAAGAATCTTTAG*	PCR, Seq
OLEC856	R	*TTTTCACGGACTCGATAC*	
OLEC857	F	*CTTCAAGTTCTGTTTCC*	
OLEC858	R	*ATATGCGTTAAAAGTTAG*	
OLEC869	F	*GCTCTTATTCCTTGACGC*	SB probe
OLEC870	R	*AAAATCAGCACCTGAGAC*	
OLEC871	R	*TGGTTCTCCCTTAATTAC*	
OLEC872	F	*CGTTGACCTTCTTCGACG*	
OLEC968	F	ACTGAGGGATCC*AAAGAATCTTTAGTAAAGTTAGCAC*	ClpC complementation
OLEC969	R	ACTGAGGGATCC*AATTTAATGGCAACTATTGAAGAC*	

italic, sequence annealing to the template; underlined, restriction site.
F, forward primer; R, reverse primer.
SB, probe for Southern blot; NB, probe for northern blot, Seq: sequencing

Table 4: Oligonuclotides for Northern blot probes

Probes for Northern blot				
clp genes				
clpC	OLEC969	*GCTCTTATTCCTTGACGC*	F	NB probe
	OLEC970	*AAAATCAGCACCTGAGAC*	R	NB probe
clpE	OLEC973	*AGGCAAGCAAGTGATTCG*	F	NB probe
	OLEC974	*AACAGAAGGTTCATCAAC*	R	NB probe
clpX	OLEC847	*TGACTGAAGCAGGGTATG*	F	NB probe
	OLEC850	*GATCTCCTCGATGCCATC*	R	NB probe
clpP	OLEC877	*GCTCTTATTCCTTGACGC*	F	NB probe
	OLEC878	*GATGTTCTGCTGCGATAG*	R	NB probe
Virulence genes				
emm	oliRN133	*TATTCGCTTAGAAAATGAA*	F	NB probe
	oliRN134	*GCAAGTTCTTCAGCTTGTTT*	R	NB probe
ska	oliRN264	*TTGTTGTAGAGTAGTTTAGC*	F	NB probe
	OLEC636	*CGTGACTCCTCAATCGTCAC*	R	NB probe
speC	OLIRN47	*CATAATTACAGTCACTGATT*	F	NB probe
	OLIRN48	*ATCGAAATGACTAAAGTTCTTCAT*	R	NB probe
sic	OLEC1534	*GAAGAATGGCCTGAAG*	F	NB probe
	OLEC1535	*CCCTGTACCTAATGCTCC*	R	NB probe
speB	OLIRN45	*GATAACCATACGATTCAGCT*	F	NB probe
	OLIRN46	*TCTGTGTCTGATGGATAGC*	R	NB probe
slo	OLIRN49	*TGCCACTAGAATCTGCAGAAAA*	F	NB probe
	OLIRN50	*CCCAAAGGATTTCATATTGAGC*	R	NB probe
sagA	OLIRN137	*TTATTTACCTGGCGTATAAC*	F	NB probe
	OLIRN138	*ATTAGATAAGGAGGTAAACC*	R	NB probe
speI	OLEC1151	*GGAAAAATAAATGAAGGTCC*	F	NB probe
	OLEC1552	*CAAAGCCAGATTGAAATC*	R	NB probe
Regulator genes				
rgg	OLEC278	*TAGACAACTTGAATGTCAAT*	F	NB probe
	OLEC279	*AAGCTTTATCATACTCTTGT*	R	NB probe
covR	OLEC1541	*ACTGCTTTGGAAAAAGAGTTTG*	F	NB probe
	OLEC1542	*CATGACACGATTCATATTAGTC*	R	NB probe
fasA	OLEC184	*AGAGACTAGAATACCATG*	F	NB probe
	OLEC185	*TTTGACTCACGACTAGAC*	R	NB probe
fasX	OLEC182	*GAGCAATAACATTTTAGG*	F	NB probe
	OLEC183	*TTACAATCAGCTGATGTG*	R	NB probe
mga	OLIRN135	*AGCTCAGTTAACTTGATTCG*	F	NB probe
	OLIRN136	*AAAGGCGTAGACAATTGG*	R	NB probe
perR	OLEC1549	*GAATTACAGAGACACGTAAAG*	F	NB probe
	OLEC1550	*CCTGTTTGCTCATGGGCTTC*	R	NB probe
codY	OLEC274	*ATAGCCTAGAAACAGAATTA*	F	NB probe
	OLEC275	*TTCTGAATAAGAAAGCGTAT*	R	NB probe
rofA	OLEC1547	*GCATTGATTCCTTTATTAA*	F	NB probe
	OLEC1548	*CTGCTGAAAAGTTTAGTTGG*	R	NB probe
srv	OLEC276	*ACATTTTGCAACGGTATATT*	F	NB probe
	OLEC277	*AATAGGGTCATTAAGTCATA*	R	NB probe
rivR	OLEC1545	*CGGAGATGAACTTAAAATACG*	F	NB probe
	OLEC1546	*CTGAAGACGATGGATTAG*	R	NB probe
malR	OLEC1543	*GTCGACTATTACTAACGAAG*	F	NB probe
	OLEC1544	*CCATCAATATTTCTACGC*	R	NB probe
sigX	OLEC1532	*GTCGATAGAGACAAGAGC*	F	NB probe
	OLEC1533	*TCTTCATATGCTAACTTATGG*	R	NB probe

Verification of plasmid constructs by PCR, Southern blot or sequencing analysis				
ColE1	OLEC1560	*GAAGTACATCCGCAACTGTC*	F	PCR, SB, SEQ
	OLEC1562	*GTGATGCTCGTCAGGGGG*	R	PCR, SB, SEQ
repAts	oliRN228	*GGAACGAAAACTCACGTTAA*	F	PCR, SB, SEQ
	oliRN229	*AGGTTCTTGATGCTGAAACG*	R	PCR, SB, SEQ

italic, sequence annealing to the template; underlined, restriction site.
F, forward primer; R, reverse primer.
SB, probe for Southern blot; NB, probe for northern blot, SEQ: sequencing

Fig. S1: Alignment of amino acids sequence of ClpC from Gram-positive pathogens. The important amino acids from the zinc finger binding domain (purple), the Walker A (yellow) and Walker B (blue) motifs, the pore forming loop (green) and the P-loop (red) are highlighted.

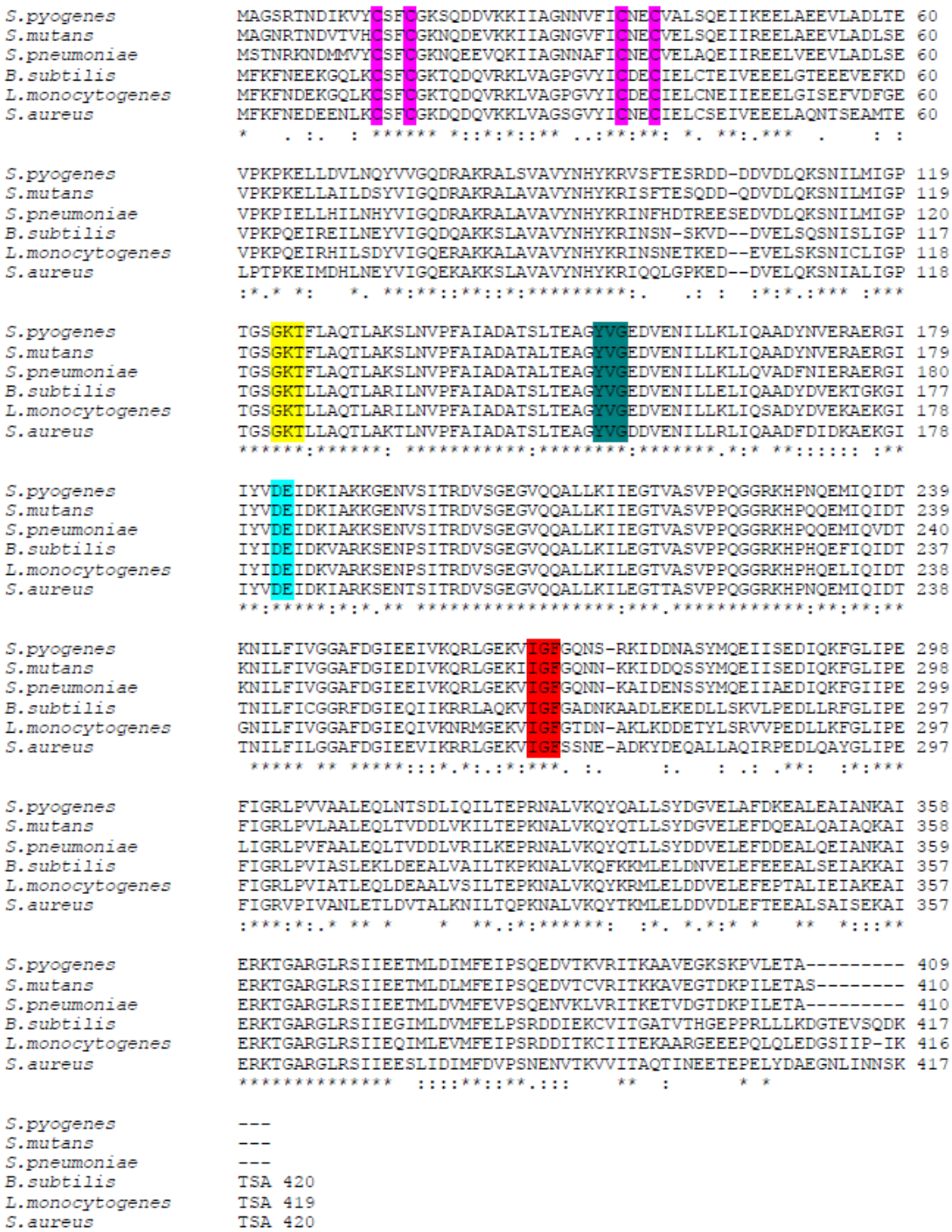


Fig. S2: Alignment of amin -acids sequence of ClpC from Gram-positive pathogens. The amino acids involved in the catalytic site are marked in green.

Fig. S3: Alignment of amino acids sequence of ClpE from Gram-positive pathogens. The important amino acids from the zinc finger binding domain (purple), the Walker A (yellow) and Walker B (blue) motifs, the pore forming loop (green) and the P-loop (red) are highlighted.

```
S.pyogenes          MLCQNCNLNESTIHLYTSVNGKQRQVDLCQNCYQIMKSDPANSILNGLTPGYRAQDRSTS 60
S.pneumoniae        MLCQNCKINDSTIHLYTNLNGKQKQIDLCQNCYKIIKTDPNNSLFKGMTD---LNNRDFD 57
B.subtilis          MRCQHCHQNEATIRLNMQINSVHKQMVLCETCYNELTRKPSMSMG----------PQSFG 50
L.monocytogenes     MNCEKCNQNPATIQLYMNINGKRVEMPLCASCYAEVRNQANFGSNE--------FPGASG 52

S.pyogenes          PFFDDFFGDLNNFRAFGNLPNTPPTQAGQNGNGGGRYGGNYNGQRPAQPQTPNQQAKGLL 120
S.pneumoniae        P-FGDFFNDLNNFRPSSNTPPIPPTQSGGGYGGNGGYGSQNRG--SAQTPPPSQ-EKGLL 113
B.subtilis          FPFEQAFQPKEQSAAKQSEKK-----------------------------------GLL 74
L.monocytogenes     SPFDDIFRQLSGAANQANREQRSQANAQVQTQTAGGG------------------NGIL 93

S.pyogenes          EEFGINVTDIARNGNIDPVIGRDEEITRVIEILNRRTKNNPVLIGEPGVGKTAVVEGLAQ 180
S.pneumoniae        EEFGINVTEIARRGDIDPVIGRDDEIIRVIEILNRRTKNNPVLIGEPGVGKTAVVEGLAQ 173
B.subtilis          DELAQNITNGAKAGLIDPVIGRDDEVARVIEILNRRNKNNPVLIGEPGVGKTAIAEGLAL 134
L.monocytogenes     DEFGTNLTDMAKNEQLDPVIGRDKEIKRVIEILNRRNKNNPVLIGEPGVGKTAVVEGLAN 153

S.pyogenes          KIIDGTVPQKLQGKQVIRLDVVSLVQGTGIRGQFEERMQKLMEEIRNRKDVILFIDEIHE 240
S.pneumoniae        KIVDGDVPHKLQGKQVIRLDVVSLVQGTGIRGQFEERMQKLMEEIRKREDIILFIDEIHE 233
B.subtilis          KIAEGDVPNKLKNKELYLLDVASLVANTGIRGQFEERMKQLITELKERKNVILFIDEIHL 194
L.monocytogenes     AIVAGDVPSKLMNKEVILLDVASLVSGTGIRGQFEERMKQLIKELQERKNTILFIDEVHT 213

S.pyogenes          IVGAGSAGDGNMDAGNILKPALARGELQLVGATTLNEYRIIEKDAALERRMQPVKVDEPS 300
S.pneumoniae        IVGAGSASDGNMDAGNILKPALARGELQLVGATTLNEYRIIEKDAALERRMQPVKVDEPT 293
B.subtilis          LVGAGSA-EGSMDAGNILKPALARGELQVIGATTLKEYRQIEKDAALERRFQPVMVQEPS 253
L.monocytogenes     IVGAGSA-EGSMDAGNILKPALARGDLQMIGATTLKEYRTIEKDAALERRFQPVTVSEPS 272

S.pyogenes          VEETITILKGIQPKYEDYHHVKYSPAAIEAAAHLSNRYIQDRFLPDKAIDLLDEAGSKMN 360
S.pneumoniae        VDETITILKGIQKKYEDYHHVQYTDAAIEAAATLSNRYIQDRFLPDKAIDLLDEAGSKMN 353
B.subtilis          IEQAILILQGIKDKYEAYHGVTFSDEAIKACVTLSSRYIQDRHLPDKAIDLLDEAGSKAN 313
L.monocytogenes     TKETLTILNGLKPKYEDFHEVVYSPEALTAAVELSARYIQDRHLPDKAIDLMDEVGSKYN 332

S.pyogenes          LTLNFVDPKEIDKRLIEAENLKAQATRDEDYERAAYFRDQITKYKEMQAQKVDEQDIPII 420
S.pneumoniae        LTLNFVDPKVIDQRLIEAENLKSQATREEDFEKAAYFRDQIAKYKEMQKKKITDQDTPSI 413
B.subtilis          LLIDELNDEDAAERLTAIEAEKTKALEEENYELAAKLRDEELALEKKL-NSSSAHTAVTV 372
L.monocytogenes     LSIEKLDENTVSERVARLEDEKNQALQMEDYEKAAKVRDEITRLEENK-TSNSFSERPVI 391

S.pyogenes          TEKTIEAIVEQKTNIPVGDLKEKEQSQLVNLANDLKAHVIGQDDAVDKIAKAIRRNRVGL 480
S.pneumoniae        SEKTIEHIIEQKTNIPVGDLKEKEQSQLIHLAEDLKSHVIGQDDAVDKIAKAIRRNRVGL 473
B.subtilis          EAEHIQEIVEQKTGIPVGKLQADEQTKMKELEAKLHERVIGQEAAVQKVAKAVRRSRAGL 432
L.monocytogenes     QASDIQAIIEEKTGIPVGRLQEDEQSKMKNLERNLTGKVIGQEDAVKKVAKAIRRSRVGL 451

S.pyogenes          GTPNRPIGSFLFVGPTGVGKTELSKQLAIELFGSTNNMIRFDMSEYMEKHAVAKLVGAPP 540
S.pneumoniae        GTPNRPIGSFLFVGPTGVGKTELSKQLAIELFGSADSMIRFDMSEYMEKHSVAKLVGAPP 533
B.subtilis          KSKNRPVGSFLFVGPTGVGKTELSKTLADELFGTKDAIIRLDMSEYMEKHAVSKIIGSPP 492
L.monocytogenes     KSKNRPIGSFLFVGPTGVGKTELGRTLARELFGTSEAMIRLDMSEFMEKHSISKLIGSPP 511

S.pyogenes          GYIGYEEAGQLTEQVRRNPYSLILLDEVEKAHPDVMHMFLQVLDDGRLTDGQGRTVSFKD 600
S.pneumoniae        GYVGYDEAGQLTEKVRHNPYSLILLDEVEKAHPDVMHMFLQVLDDGRLTDGQGRTVSFKD 593
B.subtilis          GYVGHEEAGQLTEKVRRNPYSIVLLDEIEKAHPDVQHMFLQIMEDGRLTDSQGRTVSFKD 552
L.monocytogenes     GYVGHEEAGQLTEKVRRNPYSIILLDEIEKAHPDVQHMFLQILEDGRLTDSQGRTVSFKD 571

S.pyogenes          TIIIMTSNAGTGKSEASVGFGAAREGRT---SSVLGELSNFFSPEFMNRFDGIIEFKALS 657
S.pneumoniae        AIIIMTSNAGTGKTEASVGFGAAREGRT---NSVLGELGNFFSPEFMNRFDGIIEFKALS 650
B.subtilis          TVIIMTSNAGAGEKQTKVGFQSD-DSVIEEQT-LIDSLSMFFKPEFLNRFDSIIEFRSLE 610
L.monocytogenes     TVIIMTSNAGATDTEASVGFNTTTETKLEKGSDILAKLGAYFKPEFLNRLDSVIEFKSLE 631

S.pyogenes          KEHLLHIVDLMLEDVNERLGYNGIHLDVTQKVKEKLVDLGYDPKMGARPLRRTIQDYIED 717
S.pneumoniae        KDNLLQIVELMLADVNKRLSSNNIRLDVTDKVKEKLVDLGYDPKMGARPLRRTIQDYIED 710
B.subtilis          KEHLVKIVSLLLGELEETLAERGISLNVTDEAKEKIAELGYHPSFGARPLRRTIQEWVED 670
L.monocytogenes     KDDLVQIIDLMLVDLNEMLAQEGVTIDVSKEVKEHLIDLGYDPKFGARPLRRTIQEHLED 691

S.pyogenes          AITDYYLEHPTEKQLRALMTNSENITIKAVKEGDSFLNEESLD 760
S.pneumoniae        TITDYYLENPSEKDLKAVMTSKGNIQIKSAKKAEVKSSEKEK- 752
B.subtilis          EMTDLLLDNGEITSFHVILEDDK-IKVRAK------------- 699
L.monocytogenes     TIADSLIDQPEAKNLVATLNDNKEITITEQVTA---------- 724
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Fig. S4: Alignment of amino acids sequence of ClpC from Gram-positive pathogens. The important amino acids from the Walker A (yellow) and Walker B (blue) motifs, the pore forming loop (green) and the P-loop (red) are highlighted.

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B.subtilis          MMFGRFTERAQKVLALAQEEALRLGHNNIGTEHILLGLVREGEGIAAKALQALGLGSEKI 60
L.monocytogenes     MMFGRFTQRAQKVLALSQEEAMRLNHSNLGTEHILLGLVREGEGIAAKALYELGISSEKV 60
S.pyogenes          MIM--YSTKMQDIFRQAQFQAARFDSHCLETWHVLLAMVAVDNSLANMILSEYDAQVAIE 58
S.pneumoniae        -MN--YSKALNECIESAYMVAGHFGARYLESWHLLIAMSNHSYSVAGATLNDYPYEMDRL 57
S.aureus            -----------------MNNGFFNSDFDSIFRRMMKDMQGSNQVGNKKYYINGKEVS-- 40

B.subtilis          QKEVESLIGRGQ---EMSQTIHY---TPRAKKVIELSMDEARKLGHSYVGTEHILLGLIR 114
L.monocytogenes     QQEVEGLIGHGE---KAVTTIQY---TPRAKKVIELSMDEARKLGHTYVGTEHILLGLIR 114
S.pyogenes          EYEAAAILAMGKTPKEQLSRVDFRPQSKTLTNLLAFAQAISQITRDQEVGSEHVLFAILL 118
S.pneumoniae        EEVALELTETDYSQDETFTELPF---SRRLQVLFDEAEYVASVVHAKVLGTEHVLYAILH 114
S.aureus            PEELAQLTQQGG------------------------------------------------ 52

B.subtilis          EGEGVAARVLNNLGVSLN--------------------------KARQQVLQLLGSNET 147
L.monocytogenes     EGEGVAARVLSNLGISLN--------------------------KARQQVLQLLGGGDA 147
S.pyogenes          NPDIMASRLLEIAGYQIKDNGNGQPRLADLRKAIERHAGYSKEMIKAIHELRKPKKTKTQ 178
S.pneumoniae        DSNALATRILERAGFSYEDK-KDQVKIAALRRNLEERAGWTREDLKALRQRHRTVADKQN 173
S.aureus            ----------------------------------------------NHSAEQSAQAFQQ 65

B.subtilis          GSSAAGTNSNANTPTLDSLARDLTAIAKEDSLDPVIGRSKEIQRVIEVLSRRTKNNPVLI 207
L.monocytogenes     TGAGRQTNTQA-TPTLDSLARDLTVIAREDNLDPVIGRSKEIQRVIEVLSRRTKNNPVLI 206
S.pyogenes          GTFSDMMKPPSTAGELSDFTRDLTEMARQGLLESVIGRDQEVSRMIQVLSRKTKNNPVLV 238
S.pneumoniae        SMANMMGMPQTPSGGLEDYTHDLTEQARSGKLEPVIGRDKEISRMIQILSRKTKNNPVLV 233
S.aureus            AAQRQQGQQGGNGNYLEQIGRNLTQEARDGLLDPVIGRDKEIQETAEVLSRRTKNNPILV 125

B.subtilis          GEPGVGKTAIAEGLAQQIINNEVPEILRDKRVMTLDMGTVVAGTKYRGEFEDRLKKVMDE 267
L.monocytogenes     GEPGVGKTAIAEGLAQQIVRNEVPETLRGKRVMTLDMGTVVAGTKYRGEFEDRLKKVMDE 266
S.pyogenes          GDAGVGKTALAYGLAQRIANGAIPYELKEMRVLELDMMSVVAGTRFRGDFEERMNQIIDD 298
S.pneumoniae        GDAGVGKTALALGLAQRIASGDVPAEMAKMRVLELDLMNVVAGTRFRGDFEERMNNIIKD 293
S.aureus            GEAGVGKTAIVEGLAQAIVEGNVPAAIKDKEIISVDISSLEAGTQYRGAFEENIQKLIEG 185

B.subtilis          IRQAGNIILFIDELHTLIGAG---GAEGAIDASNILKPSLARGELQCIGATTLDEYRKYI 324
L.monocytogenes     IRQAGNVILFIDELHTLIGAG---GAEGAIDASNILKPPLARGELQCIGATTLDEYRKYI 323
S.pyogenes          IEADGQIILFVDELHTIMGSGS--GIDSTLDAANILKPALSRGTLHMVGATTQEEYQKHI 356
S.pneumoniae        IEEDGQVILFIDELHTIMGSGS--GIDSTLDAANILKPALARGTLRTVGATTQEEYQKHI 351
S.aureus            VKSSQNAVLFFDEIHQIIGSGATGSDSGSKGLSDILKPALSRGEISIIGATTQDEYRNNI 245

B.subtilis          EKDAALERRFQPIQVDQPSVDESIQILQGLRDRYEAHHRVSITDDAIEAAVKLSDRYISD 384
L.monocytogenes     EKDAALERRFQPIKVDEPTVEESIQILHGLRDRYEAHHRVAITDEALEAAVRLSDRYISD 383
S.pyogenes          EKDAALSRRFAKILIEEPNTEDAYQILMGLKLSYETYHNVSISNEAVKTAVKMAHRYLTS 416
S.pneumoniae        EKDAALSRRFAKVTIEEPSVADSMTILQGLKATYEKHHRVQITDEAVETAVKMAHRYLTS 411
S.aureus            LKDAALTRRFNEVLVNEPSAKDTVEILKGIREKFEEHHQVKLPDDVLKACVDLSIQYIPQ 305

B.subtilis          RFLPDKAIDLIDEAGSKVRLRSFTTPPNLKELEQKLDEVRKEKDAAVQSQEFEKAASLRD 444
L.monocytogenes     RFLPDKAIDVIDESGSKVRLKSFTTPKNVKEMENNLSDLKKEKDAAVQGQEFEKAASLRD 443
S.pyogenes          KNLPDSAIDLLDEASAAVQN----MVK--KSAPETLTPI----DQALINGDMKKVSRLLA 466
S.pneumoniae        RHLPDSAIDLLDEAAATVQN----KAKHVKADDSDLSPA----DKALMDGKWKQAAQLIA 463
S.aureus            RLLPDKAIDVLDITAAHLSAQS--PAVDKVETEKRISELENDKRKAVSAEEYKKADDIQN 363

B.subtilis          TEQRLREQVEDTKKSWKEKQGQENSEVTVDDIAMVVSSWTGVPVSKIAQTETDKLLNMEN 504
L.monocytogenes     KEQKLKKSLEETKANWQEKQGLDHSEVTEDIVAEVVASWTGIPVAKLAETETNKLLNMEK 503
S.pyogenes          KEA--------------KGQMRKPTPVTEDDILATLSKLSGIPLEKLTQADSKKYLNLEK 512
S.pneumoniae        KEE--------------EVPVYK-DLVTESDILTTLSRLSGIPVQKLTQTDAKKYLNLEA 508
S.aureus            EIKSLQDKLE-------NSNGEHTAVATVHDISDTIQRLTGIPVSQMDDNDIERLKNISN 416

B.subtilis          ILHSRVIGQDEAVVAVAKAVRRARAGLKDPKRPIGSFIFLGPTGVGKTELARALAESIFG 564
L.monocytogenes     LLHERVIGQDAAVKAVSLAVRRARAGLKDPKRPIGSFIFLGPTGVGKTELARALAESMFG 563
S.pyogenes          ELHKRVIGQDAAVTAISRAIRRNQSGIRTGKRPIGSFMFLGPTGVGKTELAKALAEVLFD 572
S.pneumoniae        ELHKRVIGQDQAVSSISRAIRRNQSGIRSHKRPIGSFMFLGPTGVGKTELAKALAEVLFD 568
S.aureus            RLRSKIIGQDQAVEMVSRAIRRNRAGFDDGNRPIGSFLFVGPTGVGKTELAKQLAIDLFG 476

B.subtilis          DEESMIRIDMSEYMEKHSTSRLVGSPPGYVGYDEGG-QLTEKVRRKPYSVVLLDEIEKAH 623
L.monocytogenes     DEDSMIRIDMSEYMEKFSTARLVGAPPGYVGYEEGG-QLTEKVRQKPYSVVLLDEIEKAH 622
S.pyogenes          DEAALIRFDMSEYMEKFAASRLNGAPPGYVGYDEGG-ELTQKVRNKPYSVLLFDEVEKAH 631
S.pneumoniae        DESALIRFDMSEYMEKFAASRLNGAPPGYVGYEEGG-ELTEKVRNKPYSVLLFDEVEKAH 627
S.aureus            NKDALIRLDMSEYSDTTAVSKMIGTTAGYVGYDDNSNTLTEKVRRNPYSVILFDEIEKAN 536
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B.subtilis           PDVFNILLQVLEDGRLTDSKGRTVDFRNTILIMTSNVGASELKRNKYVGFNVQDETQNHK 683
L.monocytogenes      PDVFNMLLQVLDDGRLTDSKGRVVDFRNTVIIMTSNIGAQEMKQDKSMGFNVTDPLKDHK 682
S.pyogenes           PDIFNVLLQVLDDGILTDSRGRKVDFSNTIIIMTSNLGATALRDDKTVGFGVKDIHQDHQ 691
S.pneumoniae         PDIFNVLLQVLDDGVLTDSKGRKVDFSNTIIIMTSNLGATALRDDKTVGFGAKDIRFDQE 687
S.aureus             PQILTLLLQVMDDGNLTDGQGNVINFKNTIIICTSNAGFGNGNDAE-------------- 582

B.subtilis           DMKDKVMGELKRAFRPEFINRIDEIIVFHSLEKKHLTEIVSLMSDQLTKRLKEQDLSIEL 743
L.monocytogenes      AMEHRVLQDLKQAFRPEFINRIDETIVFHSLQEKELKQIVTLLTAQLTKRLAERDIHVKL 742
S.pyogenes           AMEKRILEELRKTYRPEFINRIDEKVVFHSLTQDNMRDVVKIMVQPLITTLAEKGITLKI 751
S.pneumoniae         NMEKRMFEELKKAYRPEFINRIDEKVVFHSLSSDHMQEVVKIMVKPLVASLTEKGIDLKL 747
S.aureus             --EKDIMHEMKKFFRPEFLNRFNGIVEFLHLDKDALQDIVNLLLDDVQVTLDKKGITMDV 640

B.subtilis           TDAAKAKVAEEGVDLEYGARPLRRAIQKHVEDRLSEELLRGNIHKGQHIVLDVEDGEFVV 803
L.monocytogenes      TEGAKSKIAKDGYDPEYGARPLKRAIQKEVEDMLSEELLRGNIKVGDYVEIGVKDGKLEV 802
S.pyogenes           QPLALKHLSEVGYDEHMGARPLRRTLQTEIEDKLSELILSRELTSGHTLKIGLSHGKLTF 811
S.pneumoniae         QASALKLLANQGYDPEMGARPLRRTLQTEVEDKLAELLLKGDLVAGSTLKIGVKAGQLKF 807
S.aureus             SQDAKDWLIEEGYDEELGARPLRRIVEQQVRDKITDYYLDHTDVK--HVDIDVEDNELVV 698

B.subtilis           K--------TTAKTN--- 810
L.monocytogenes      RKKDAPKKKTTSKKVKAK 820
S.pyogenes           HIA--------------- 814
S.pneumoniae         DIA--------------- 810
S.aureus             KGK--------------- 701
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Curriculum Vitae

Surname	Beneyt
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Date of Birth	14th October 1982 (28 years old)
Place of Birth	Paris, France
Nationality	French
Family status	Single

Education

Jun 2000	Baccalaureat (A-level): Biology, Physics, Chemistry, and Mathematics
Sep.2000-Jun.2001	First year Medicine study at the University of Paris VI
Sep.2001-Jun.2004	Bachelor of Genetics (third year degree) at the University Paris VII (France)
Jun.2004-Sep.2004	Training period at the University of Leicester (England), Dept of Microbiology, in Prof. J. Ketley group Subject: **Complementation of a *chuA* mutant, an iron carrier in *Campylobacter jejuni.***
Sep.2004-Jun.2006	Master study in Genetics at the University of Paris VII (France)
Mar.2005-Sep.2005	Training period the SCRIPPS institute (La Jolla, California), Dept. of Oncology, in Prof. D.E. Zhang group Subject: **Modification of HSPC150 by ISG15, an ubiquitin family member**
Dec.2005-Jun.2006	Master thesis at Necker Medical School (Paris, France) in Dr JM. Reyrat group Title: **Species specificity of a glycopeptidoprotein carrier (Gap) in *Mycobacterium***
Jun.2006	Master Degree in Genetics at the University of Paris VII (France)
Oct.2006-Oct.2009	PhD Thesis at the Max F. Perutz Laboratories (Vienna, Austria), Institute of Microbiology and Immunobiology, in Dr. E. Charpentier group Title: **Role of Clp-mediated proteolysis in the gram positive pathogen *Streptococcus pyogenens***
Nov.2009-Jul.2010	Relocation of research at the University of Umea (Sweden), Dept. of Molecular Biology, MIMS, in Dr E. Charpentier group

Scientific experience

- S2 Lab, Sterile hood
- Writing Reports, Presentations

Molecular Biology:

- DNA: Cloning, Southern blot
- RNA: Northern blot, Reverse transcription
- Protein: Purification, Overexpression, Western blot
- Cell Culture : Culture, Transfection, Infection

Microbiology:

- *S. pyogenes, S. aureus, M. smegmatis, C. jejuni, E. coli*
- Knock out strains construction in *S. pyogenes* and *S. aureus*
- Biofilm assay, Autolysis assay, Motility, Survival to stress, Infection
- Light and Scanning Electron Microscopy

Teaching

- Tutor for "UE in Molekularer Mikrobiologie" University of Vienna (2008)
- Tutor for the supervision of exams of Molecular Biology study (2007-2009)
- Supervision of 2 praktikum students and 1 diploma student

Business

- Selling at C&A Haussmann (Paris, France), Men's department (2001-2002)
- Cashier at C&A Haussmann (Paris, France) (2003)

Languages

- **French**: Native speaker
- **English**: Fluent
- **German**: Advanced (B2)
- **Spanish**: Conversational

Further informations

- Computer skills: PC and Mac, Office (Word, Excel, Powerpoint), Photoshop, VectorNTI, DNA Star
- Driving licence B
- Golf, Swimming, Ball room dancing, Badminton
- Cinema, Live music, French and American literature

Oral presentation

- 18.11.2008: VBC symposium II, Intercell, Vienna, Austria.
  Fanny Beneyt: **Role of Clp mediated proteolysis in *Streptococcus pyogenes* pathogenicity**

Poster presentation

- 18.10.2007: VBC Symposium, Intercell, Vienna, Austria
  Fanny Beneyt, Silvia Spiess, Tim Clausen, Emmanuelle Charpentier
  Role of Clp protein in Gram positive pathogens

- 6-7.03.2008: 3rd Symposium of the DFG Priority program. *Proteolysis in prokaryotes: Protein quality control and regulatory principles.* Berlin, Germany
  Fanny Beneyt, Silvia Spiess, Kürsad Turgay, Tim Clausen, Emmanuelle Charpentier
  Role of Clp-mediated proteolysis in Gram positive human pathogens

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