university of groningen

University Medical Center Groningen

University of Groningen

Snap Shots of Bacterial Adaptation

Prajapati, Bimal

DOI:
10.33612/diss.145910301

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2020

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Prajapati, B. (2020). *Snap Shots of Bacterial Adaptation*. University of Groningen. https://doi.org/10.33612/diss.145910301

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 05-06-2022

Chapter 1

General Introduction and Scope of the thesis

Introduction

Since the dawn of life some 3.7 billion years ago, microbes have had to survive and adapt to their surrounding environment (Dodd et al, 2017). Changes in the environment maybe relatively subtle, as exemplified by slight variance in the temperature, pH or salinity. On the other hand, environmental changes can be more drastic with completely different surroundings, for instance when a microbe enters another organism, a type of process that has ultimately led to the evolution of organelles, especially mitochondria and chloroplasts. Likewise, the entry of a pathogen into a host cell or its engulfment by immune cells of the host represents a major environmental transformation. Apart from adapting to changes in environmental conditions, microbes have to compete for nutrients and eliminate competitors by producing toxins and antibiotics. Conversely, they also have to defend themselves from the toxins and antibiotics produced by competitors or predators. Consequently, continuous micro and macro adaptation is the way of life, and it is key for survival in ever-changing environments where only the fittest will survive (Darwin, 1859).

Transient and sustained adaptation

Bacterial adaptation can be broadly classified into two types. The first being transient adaptation that is swift and mainly occurs by modulating gene transcription and protein translation based on the actual need and stress stimuli. The second is sustained adaptation, which usually occurs via mutation or accumulation of mutations. The resulting changes in the genome will ultimately affect the gene expression and/or protein function and are usually associated with a fitness cost as a consequence of the acquired mutation. This thesis focuses on the various mechanisms that are involved in transient bacterial adaptations.

Transient adaptation in bacteria primarily involves the modulation of gene expression. Gene expression in bacteria is a highly regulated, complex and continuous process. As in all living organisms, the DNA is the template from which genes are transcribed into RNA by RNA polymerase and concurrently translated into proteins by ribosomes. The spatiotemporal sequence in which genes are transcribed and the extent of transcription are generally strictly controlled in a living cell. Accordingly, the transcription of many genes is specifically induced or repressed depending on the environmental conditions encountered by the bacteria, for example the availability of nutrients or the

presence of physical or chemical stress stimuli. Control over the gene expression in bacteria is primarily attributed to sigma factors. Sigma factors recognize promotor regions which are specific DNA sequence elements situated upstream of genes. Binding of sigma factors to the recognized promoter region allows RNA polymerase assembly and initiates the synthesis of RNA transcripts of a gene. The expression of sigma factors themselves can be regulated by specific sensing mechanisms and regulatory cascades, which often involve so-called two component regulatory systems that couple the sensing of specific stimuli to appropriate transcriptional responses (Haugen et al, 2008).

The complete set of RNA molecules produced in a cell is referred to as its transcriptome and the study of the cellular transcriptome is transcriptomics. The recent technical advances in transcriptomics have revealed a high level of complexity in the bacterial transcriptome, debunking the classical operon concept for most of the bacterial transcriptomes that have been analysed (Güell et al, 2011). The presence of internal promoters and termination signals within operons results in a plethora of different transcripts that are regulated by more than one transcription factor (Perkins, 1996; Petersohn et al, 1999; McDaniel et al, 2003). High-throughput transcript analysis has also unveiled an unexpected abundance of small RNAs (sRNAs), which account for approximately a quarter of the bacterial genome in some cases. The regulatory roles of several sRNAs have been established with some having narrow regulatory functions, while others have a global impact on the transcriptome. Yet, only a handful of the sRNAs have been characterized and the functions of most of them are yet to be elucidated. sRNAs are usually 50-200 nucleotides in length, and can be transcribed independently from protein-encoding genes (Wang et al, Storz et al, 2011; Cech et al, 2014). sRNAs can act by base-pairing with target messenger RNA (mRNAs) with which they share little or extensive complementarity and help in refining the level of gene expression. However, sRNAs can also act by directly modulating protein activity. The fact that sRNAs don't need to be translated and have the capacity to directly modulate protein activity makes sRNA regulation much faster and attractive when a rapid response is required to adapt to sudden changes in the environment or to particular chemical or physical stimuli (Storz et al, 2011; Carrier et al, Chareyre et al, 2018; Adams et al, Jørgensen et al, 2020).

Next to adaptations based on transcriptional regulation, bacteria had also additional ways to adapt to changing conditions. Interestingly, the evidence of epigenetic modifications in bacteria and their hereditary nature is ever increasing. In particular, it has been shown that adenine and cytosine of the bacterial DNA can be methylated and that the methylated DNA has important roles in chromosome partitioning, DNA replication, repair and the timing of transposition and conjugation (Casadesús et al, 2006). Also, methylated cytosine can be involved in epigenetic transcriptional regulation and the DNA methylation seems to change transcription even in a heritable manner (Blyn et al, 1990; Sohanpal et al, 2004). Likewise, there is ample evidence of bacterial mRNA processing and polyadenylation leading to various post-transcriptional modifications. Moreover, “omics” studies have revealed the presence of long untranslated regions that contain particular regulatory structures or may be involved in anti-sense gene regulation.

Transcriptomic approach to study bacterial adaptation

The study of entire transcriptomes started in the early 1990s with the advent of microarray techniques. Before this technology became available, the early studies on the transcriptome were mostly focused on individual transcripts or groups of transcripts. Using low throughput sequencing, such as Sanger sequencing, random transcripts were sequenced and the amounts of individual transcripts were quantified by Northern blotting, nylon membrane arrays, and later reverse transcriptase quantitative PCR (RT-qPCR) (Alwine et al, 1977; Becker-André et al, 1989). The earliest efforts of broad scale transcriptomic studies were based on so-called serial analysis of gene expression (SAGE). SAGE worked by Sanger sequencing of random transcripts and subsequent quantification of the sequences by matching them to known genes (Velculescu et al, 1995).

True genome-wide transcriptomic study started with the development of microarrays that allowed measurements of the relative abundance of all mRNA transcripts for the genes that were known for the investigated organism. This enabled a holistic approach for investigating adaptive responses of bacteria at an unprecedented scale. In particular, each individual microarray analysis provided a snapshot of the adaptive behavior to changes in the environmental conditions or bacterial responses to different stimuli, be it the availability of particular nutrients or the toxic effects of antibiotics.

Microarrays

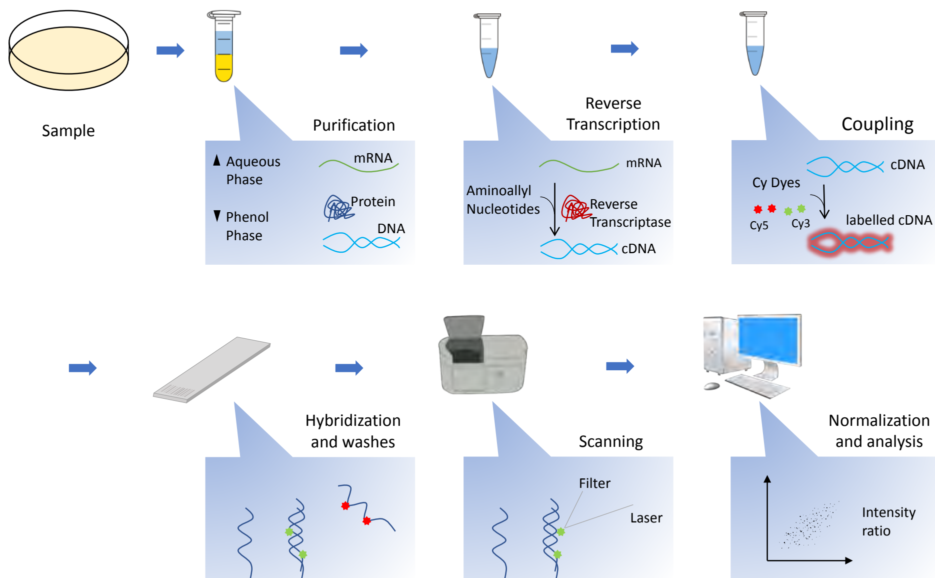


Figure 1: Schematic diagram showing the steps involved in microarray-based transcriptome analyses.

After the bacteria are grown in the desired conditions, the first step in microarray-based transcriptome analyses is to isolate and purify bacterial RNA, which is usually done by a phenol-chloroform extraction process. The purified RNA is then converted to cDNA by reverse transcription. The resulting cDNA is then labelled with dyes and loaded onto the probe-containing microchip for hybridization. After washing away unhybridized cDNA, the intensity signals are read out using a laser scanner. The obtained data is finally normalized and analyzed to give insight into the transcriptome of the bacteria at that particular time and condition.

Microarrays used for transcriptome studies consist of short nucleotide oligomers, known as "probes", which are typically arrayed in a grid on a glass slide (also known as a DNA chip) (Romanov et a, 2014). Each spot in the grid contains a known DNA sequence. When performing microarray analyses, mRNA is collected from a control and an experimental sample. The collected RNA is converted to cDNA in order to increase its stability and, at the same time, marked with fluorophores. The fluorescently labelled cDNA is spread on the surface of the microarray where it hybridizes with the corresponding probes on the grid. Using a laser, the microarray chip is scanned to quantify the fluorescence intensity of each spot on the array that corresponds to a particular gene. A single microarray usually contains enough oligonucleotide probes to represent all known genes of an organism. However, microarrays do not provide information about unknown genes as they are usually not represented in the

microarray (Heller et al, 2002; Barbulovic-Nad et al, 2006). However, the latter limitation is overcome by the use of so-called tiling arrays, where the entire genome of an organism can be represented in the form of overlapping arrayed oligonucleotides (Nicolas et al, 2012).

Notably, transcriptomic analyses based on microarrays suffer from several limitation, like cross-hybridization artifacts, poor quantification of lowly and highly expressed genes, and the need to know the genome sequence *a priori*. Because of these technical issues, transcriptomics transitioned to high throughput sequencing-based methods.

RNA-seq

With the advent of high throughput sequencing techniques, like Next-Generation Sequencing (NGS), the array-based transcriptomics methods for studying gene expression were largely overtaken by RNA-sequencing (RNA-seq). Importantly, RNA-seq reveals the presence and quantity of RNAs in a biological sample at a given time point, hence providing the complete picture of the continuously changing cellular transcriptome at a given moment in time (Wang et al, 2009; Chu et al, 2012). RNA-seq is accomplished by the reverse transcription of RNA *in vitro* and subsequent sequencing of the resulting cDNAs (Wang et al, 2009). Transcript abundance is then determined by the number of counts of each transcript.

The RNA-seq technique has, by definition, been heavily influenced by the development of high-throughput sequencing technologies (Ozsolak et al, 2010; McGettigan et al, 2013). RNA-seq leverages deep sampling of the transcriptome with many short fragments, enabling the computational reconstruction of the original RNA transcript by aligning reads to a reference genome or to each other. This approach is known as *de novo* assembly (McGettigan et al, 2013). A key advantage of RNA-seq over microarray-based methods is its high dynamic range of 5 orders of magnitude, enabling the quantification of both low-abundance and high-abundance RNAs. In addition, the nanogram amounts of RNA needed for the RNA-seq experiments are considerably lower than the micrograms of RNA needed for microarray analyses. This permits a more precise examination of cellular structures down to the single-cell level when combined with a linear amplification of cDNA (Hashimshony et al, 2012; Svensson et al 2018). Theoretically, there is no upper limit of quantification in RNA-seq, and

background noise is very low for 100 bp reads in non-repetitive regions (Wang et al, 2009).

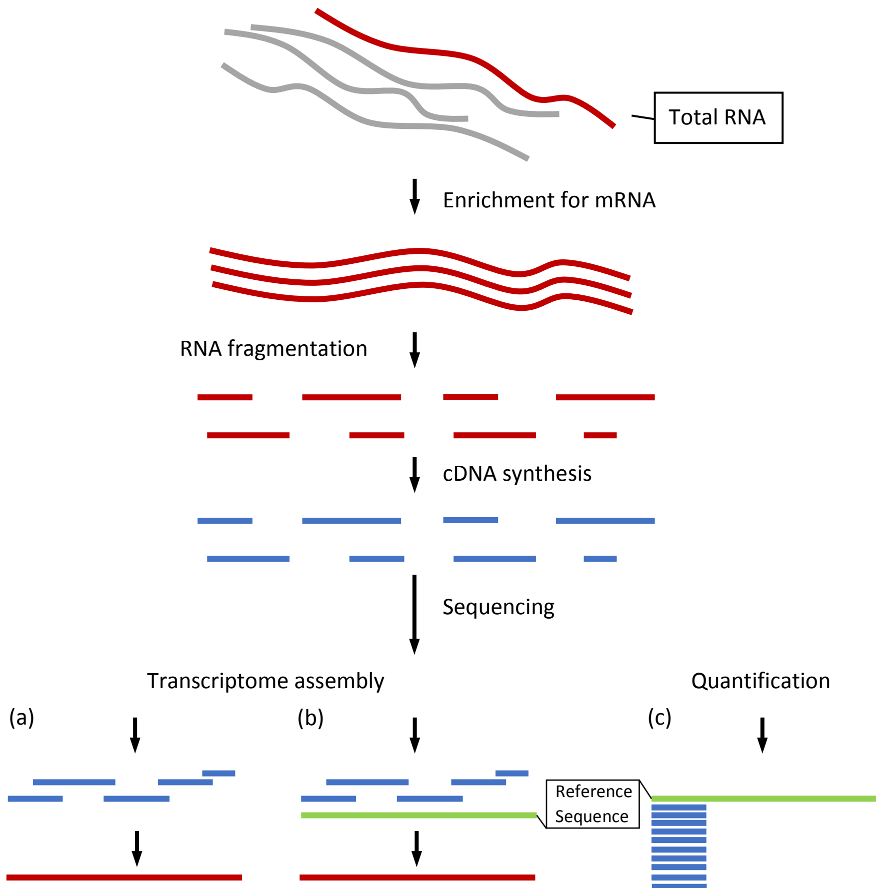


Figure 2: Schematic diagram showing the different steps involved in RNA-seq (Forde et al, 2013).

First, total RNA is extracted from the bacteria. In prokaryotes, mRNA constitutes only 1 % - 5 % of the total RNA. Therefore, after extraction of the bacterial RNA, mRNA requires enrichment prior to sequencing. Enrichment of mRNAs may include rRNA capture, processed RNA degradation and selective polyadenylation of mRNA. Following enrichment, the mRNA molecules are fragmented, then converted to cDNA and finally sequenced. The sequenced cDNA is then used for (a) *de novo* transcriptome assembly, (b) transcriptome re-sequencing or (c) transcriptome quantification.

RNA-seq data can be be exploited for various purposes, like identifying genes within a genome or just identifying genes that are active at a particular point in time. Moreover, the read counts can be used to accurately model the relative gene expression levels. Importantly, the RNA-seq technology also provides detailed information on coding and non-coding transcript structures, including transcriptional start sites, terminators and sites of RNA processing.

Mass spectrometry-based proteomics to study bacterial adaptation

The proteome is the entire set of proteins that is produced or modified by an organism or system, and the term proteomics refers to the large-scale experimental analysis of proteins and proteomes (Anderson et al, 1998; Blackstock et al, 1999). It is often used specifically to refer to protein purification and mass spectrometry. Proteomics has enabled the identification of ever-increasing numbers of proteins. The proteome varies with time and in response to distinct requirements or stresses, that a cell or organism undergoes.

There are essentially two mass spectrometry-based methods currently used for protein profiling. The classical approach employs high resolution, two-dimensional electrophoresis to separate proteins from different samples in parallel. This is followed by gel staining and selection of differentially expressed proteins to be identified by mass spectrometry. Despite the advances in two-dimensional electrophoresis and its maturity, it has its limitations. The chief concern is the inability to resolve all the proteins within a sample, given their dramatic range in expression level and differing properties. In particular, two-dimensional gel electrophoresis fails to separate hydrophobic membrane proteins, and proteins with extremely low or high pI's or Mw's. Another disadvantage of this approach is that it is, at best, semi-quantitative.

Compared to the classical two-dimensional gel-based proteomics approaches, a wide range of partially gel-based or completely gel-free proteomics approaches has been developed in recent years. These are superior with respect to the quantification of relative protein amounts, and may even allow absolute protein quantifications (Antelo-Varela et al, 2019; 2020). A quantitative approach that allows the precise comparison of proteins in different samples uses stable isotope tags. In this method, the proteins of cells grown under different conditions are first labelled with different isotopes. Subsequently, the samples with differentially labelled proteins are mixed and then digested with trypsin to obtain labelled peptides that are separated by multidimensional liquid chromatography and finally analysed by tandem mass spectrometry.

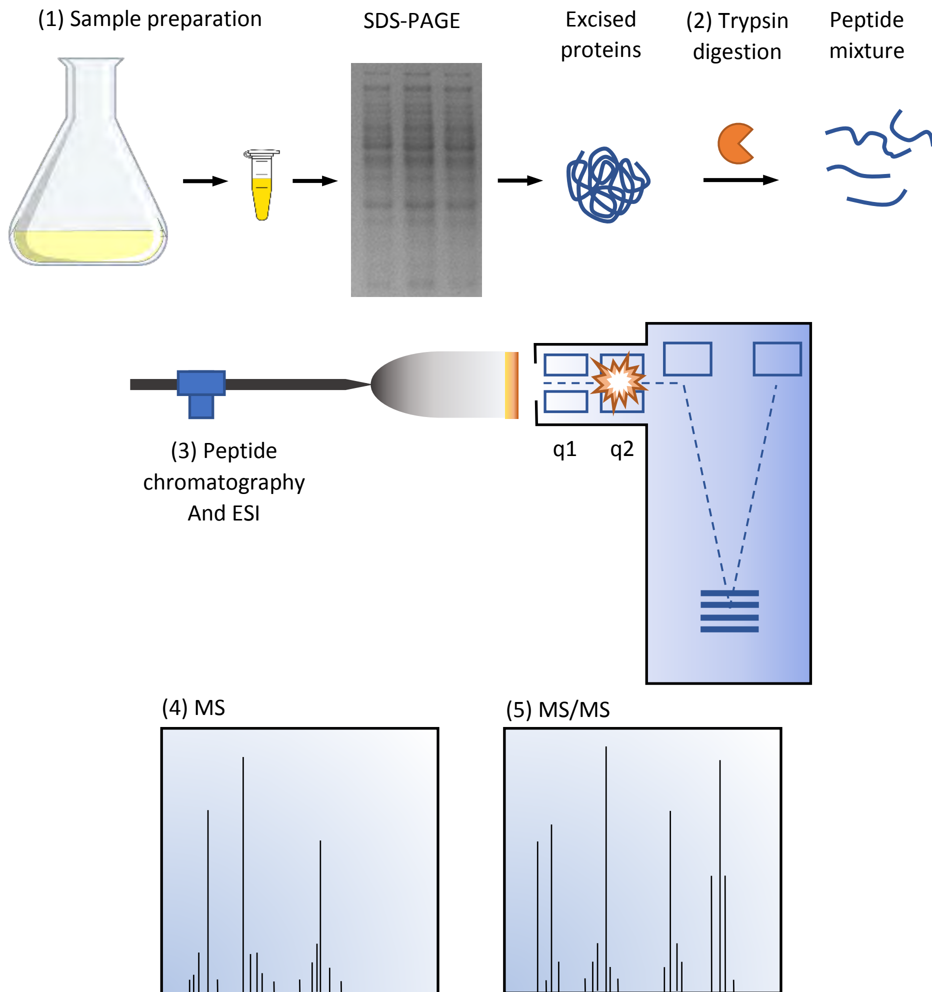


Figure 3: Schematic diagram representing the different steps in mass-spectrometry based proteomics (Aebersold et al, 2003).

A typical proteomics experiment usually consists of five stages. In stage 1, the proteins to be analysed are isolated from the bacterial cells. This often includes a final step of one-dimensional gel electrophoresis, and defines the 'sub-proteome' to be analysed. In stage 2, the proteins are degraded enzymatically to peptides usually by trypsin, leading to peptides with C-terminally protonated amino acids. In stage 3, the peptides are separated by one or more steps of high-performance liquid chromatography and subsequently eluted into an electrospray ion source, where they are nebulized in small, highly charged droplets. These highly charged droplets quickly evaporate and multiple protonated peptides enter the mass spectrometer. A mass spectrum of the peptides eluting at this time point is taken in stage 4. In stage 5, the computer generates a prioritized list of these peptides for fragmentation and a series of tandem mass spectrometric or 'MS/MS' experiments ensues. The MS and MS/MS spectra are matched against protein sequence databases, which helps to identify the peptides and the respective proteins that are present in the sample.

Mass spectrometry has become an important and widely used method for the accurate characterization of proteins. It has a broad range of applications that includes not only the identification of proteins and their masses, but also the definition of post-translational modifications, elucidation of protein complexes, protein subunits and functional interactions. Moreover, it can also be used for subcellular localization of proteins to the various organelles (Barrera et al and Cox et al, 2011).

During the PhD research described in this thesis, the adaptive behavior of two Gram-positive bacteria was explored by using various transcriptomic and proteomic approaches. The first one is *Bacillus subtilis*, a rod-shaped spore-forming non-pathogenic bacterium that is predominantly found in the soil, plant rhizosphere. However, *B. subtilis* is also frequently encountered in the gastrointestinal tract of ruminants and humans. The second one is *Staphylococcus aureus*, a coccoid bacterium that is a common member of the microbiota of humans and many animal species. *S. aureus* is often found in the upper respiratory tract and on the skin. Unlike *B. subtilis*, *S. aureus* is known to be an opportunistic pathogen.

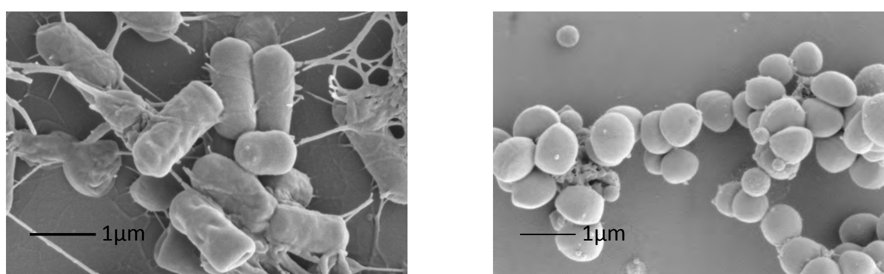


Figure 4: Scanning electron micrographs of *B. subtilis* (left panel) and *S. aureus* (right panel).

Studying adaptive processes in *Bacillus subtilis*

B. subtilis is well known for its high capacity to secrete proteins and, because of its highly efficient secretion machinery, *B. subtilis* is tremendously exploited as a biotechnological "cell factory" for the production of commercially relevant proteins such as amylases, proteases and xylanases (Harwood et al, 2007; van Dijl et al, 2013). The protein secretion by *B. subtilis* occurs via two major protein translocation pathways, namely the Sec pathway and the Tat pathway. The Sec pathway, which is also known as the general secretion pathway, involves the translocation of proteins in an unfolded state. Hence the protein folding takes place post-translocationally (Driessen et al, 2008; Yuan et al, 2010; Park et al

2012). On the other hand, the Tat secretion pathway can transport fully folded proteins across the membrane (Goosens et al, 2016; Frain et al, 2019). The Tat, or twin-arginine translocation pathway is present in bacteria, archaea and also in organelles like chloroplast thylakoids and the inner membrane of plant mitochondria (Tjalsma et al, 2004; Jongbloed et al, 2006; Palmer et al, 2012; Schäfer et al; 2020). In order for the proteins to be translocated by the Tat pathway they need to be recognized by the Tat translocase first, which requires the presence of a twin-arginine "RR"-motif in the N-terminal region of their signal peptide (Fröbel et al, 2012; Beck et al, 2013). In addition, the cargo protein must be in a folded state (Goosens et al, 2016; Frain et al, 2019).

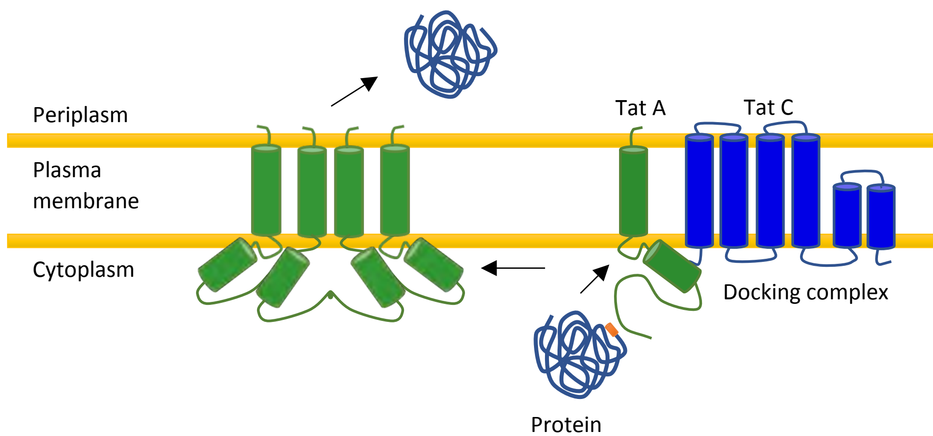


Figure 5: Schematic representation of the Tat pathway in *B. subtilis* (Goosens et al, 2014).

The Figure shows a conventional representation of the membrane topology of TatA and TatC, based on molecular biological analyses. The TatA and TatC subunits form the docking complex of the *B. subtilis* Tat translocase that serves in the reception of folded proteins with an RR-motif in their signal peptide. Once the cargo is bound by the docking complex, additional TatA subunits are recruited to form the active TatA-TatC translocase.

The *Bacillus* Tat translocase consists of so-called TatA and TatC subunits, where TatA is the smaller subunit having a single N-terminal transmembrane domain, whereas the larger TatC subunit has six transmembrane domains (Goosens et al, 2016; Frain et al, 2019). The TatA and TatC subunits together form a docking complex that recognizes and binds proteins with the correct "RR"-signal. This interaction will activate the docking complex and initiates the translocation process. The docking complex inserts the substrate into the membrane. During this step additional TatA subunits are recruited, which either results in weakening of the membrane or the formation of a pore through which the protein is translocated (Hou et al, 2018; Bernal-Cabas et al, 2020). The energy

for the translocation is provided by the proton-motive force (Mould et al, 1991; Yen et al, 2002; Palmer et al, 2005; Goosens et al, 2015). Meanwhile, the signal peptide is also cleaved by signal peptidases, allowing for the release of the protein on the other side of the membrane (Jongbloed et al, 2004; Lüke I et al, 2009; Dalbey et al, 2012; Krishnappa et al, 2013).

The sequencing of the *B. subtilis* genome has revealed three genes for TatA subunits and two genes for TatC subunits. The *tatAy-tatCy* and *tatAd-tatCd* genes are organized in operons at different genomic loci, and the *tatAc* gene is independently expressed from a third genomic locus (Jongbloed et al, 2000). Elaborate expression studies conducted in many different conditions have shown that the *tatAy-tatCy* operon and the *tatAc* gene are constitutively expressed (Nicolas et al, 2012). On the other hand, the *tatAd-tatCd* operon is only known to be expressed under conditions of phosphate limitation (Eder et al, 1996; Jongbloed et al, 2000). The two operons encode two separate Tat translocases named TatAyCy and TatAdCd. Each translocase operates independently from the other and translocates its own cargo proteins across the membrane (Eder et al, 1996; Jongbloed et al, 2000). TatAdCd is known to translocate a phosphodiesterase, PhoD, which is involved in the acquisition of phosphate and degradation of wall teichoic acid upon phosphate starvation (Goosens et al, 2014; Myers et al, 2016). TatAyCy is known to translocate three proteins, including the heme-peroxidase EfeB (Jongbloed et al, 2004; Miethke et al, 2013), the Rieske iron-sulpher protein QcrA (Goosens et al, 2013) and the metallo-phosphoesterase YkuE (Nicolas et al, 2012, Monteferrante et al, 2012; Goosens et al, 2013). The independently expressed TatAc is a dispensable subunit of the TatAyCy translocase. It is known to enhance the translocase's efficiency when the function of the TatAy subunit is compromised by mutations, but it cannot replace TatAy (Goosens et al, 2013). Hence TatAyCy is regarded as the core Tat translocase in *B. subtilis*.

Though there has been substantial progress in understanding the structure and working mechanism of the *B. subtilis* Tat translocase, there is still a huge gap in our understanding of the biological functions of the TatAyCy core translocase. While the Tat system is not essential for *B. subtilis* under most growth conditions, it was discovered that TatAyCy is very important for growth in Lysogeny Broth (LB) lacking NaCl (van der Ploeg et al, 2011). When grown in LB a defective growth phenotype. The *tat*-mutants initially start growing rapidly

without NaCl, mutant strains lacking *tatAy*, *tatCy* or both these *tat* genes show until the early exponential growth phase is reached, but then the *tat*-mutant bacteria will stop growing and lyse. Nonetheless, part of the population of mutant bacteria can adapt to the salt-deprived condition and resume growth. Previous studies attributed this growth defect of the *tat* mutants in LB without NaCl to the failed translocation of the Tat substrate EfeB, because the *efeB* mutants also portrayed a similar growth phenotype as the *tat* mutants in LB without NaCl. This substantiated the claim that failed transport of EfeB is the most likely cause for the defective growth in LB without NaCl (Goosens et al, 2015).

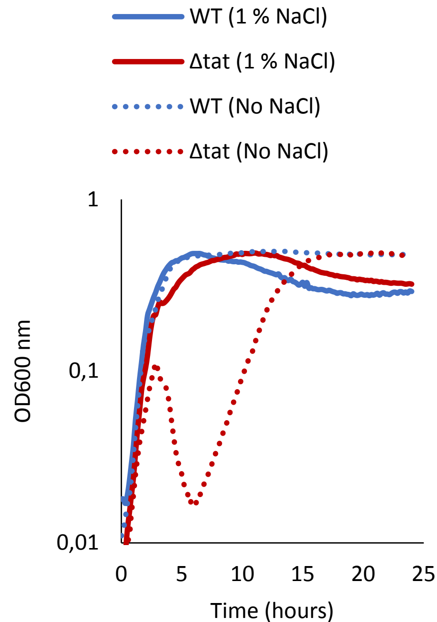


Figure 6: Growth of the *B. subtilis* wild-type (WT) strain 168 and a total-*tat1* mutant (Δ*tat*) derivative strain in LB medium with or without 1% NaCl as monitored in a microtiter plate reader.

In LB without NaCl, the Δ*tat* strain displays a lysis-recovery phenotype, which is typical for the growth of all *tat*-deficient *B. subtilis* strains (van der Ploeg et al, 2011).

The heme-dependent peroxidase EfeB is a subunit of the elemental iron transport complex EfeUOB in *B. subtilis* (Figure 7). EfeB oxidizes ferrous iron to ferric iron at the extracytoplasmic membrane surface by neutralizing the highly oxidative H_2O_2 to water and oxygen. This reaction not only provides ferrous iron for uptake by the EfeUO minimal transport unit, but also relieves oxidative stress in the membrane environment by consuming H_2O_2 and preventing the formation of reactive oxygen species by Fenton-based chemistry. The lipoprotein EfeO captures the ferric iron and transfers it to the ferric iron permease EfeU in the membrane (Miethke et al, 2013).

Part of the research described in this thesis explores the mechanisms behind the adaptation of *B. subtilis* to medium lacking NaCl, and illustrates how the Tat pathway and the efeUOB system serve in the adaptation to medium lacking NaCl.

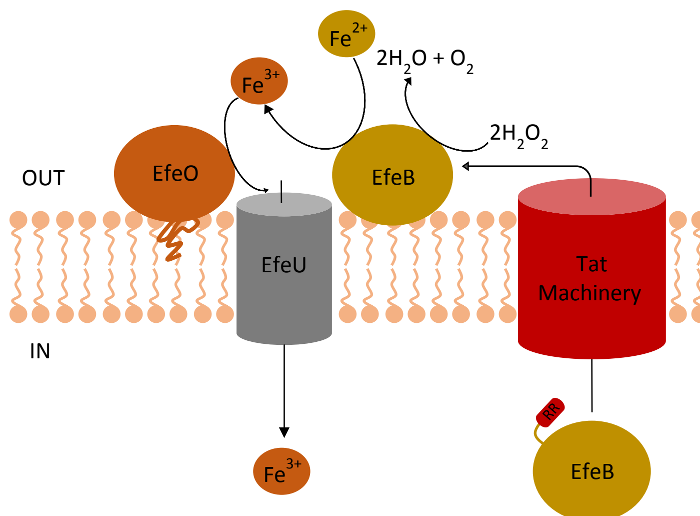


Figure 7: Schematic representation of the EfeUOB elemental iron transport system and the Tat translocase in the membrane of *B. subtilis*.

The cartoon depicts the Tat-dependent export of the ferrous iron peroxidase EfeB, which catalyzes the conversion of Fe^{2+} to Fe^{3+} at the expense of hydrogen peroxide. The newly formed Fe^{3+} is bound by the lipoprotein EfeO and transferred to the Fe^{3+} permease EfeU for uptake by the cell. EfeB and EfeO are associated with EfeU (Miethke et al, 2013).

Studying adaptive processes in *Staphylococcus aureus*

Roughly one third of the world's human population carries *S. aureus* in the anterior nares, throat and on the skin. This bacterium is transmitted by skin-to-skin contact and contact with contaminated objects. Successful transmission and colonization depend on the ability of *S. aureus* to adjust its physiology to the changing conditions, especially to insults by the human innate and adaptive immune defenses. Even despite persistent carriage, *S. aureus* behaves usually as a human commensal. However, once *S. aureus* breaches the epithelial barrier and invades the human body, it can effectively evade the host's immune defenses, causing a broad range of diseases. These include simple skin and soft tissue infections, but invasive infections can progress to life-threatening conditions, such as severe pneumonia, sepsis, osteomyelitis, toxic shock syndrome and endocarditis (Wertheim et al, 2005; Liu et al, 2011).

S. aureus has an outstanding capacity to develop resistance against antibiotics. This ability of *S. aureus* to swiftly develop resistance was discovered soon after the clinical introduction of penicillin to treat staphylococcal infections in 1940. Within a few years, resistant strains of *S. aureus* were reported (Abraham et al, 1940; Barber et al 1947). The observed resistance to penicillin resulted from the

acquisition of a plasmid carrying the *blaZ* gene, which encodes a penicillinase that hydrolyzes the β-lactam ring of penicillin, thereby inactivating it (Hackbarth et al, 1993). With the rise of penicillin resistance, methicillin a semisynthetic derivative of penicillin was introduced in 1961. However, within a year of its introduction, methicillin resistant *S. aureus* (MRSA) strains emerged (Jevons et al, 1961). The resistance to methicillin was attributed to the acquisition of the *mecA* gene, encoding the penicillin-binding protein (PBP) 2a. Expression of PBP 2a, also known as PBP2, is responsible for resistance to a wide range of β-lactam antibiotics, including methicillin. This relates to the fact that PBP2 has a lowered affinity for β-lactam antibiotics, allowing the synthesis of the bacterial cell wall in spite of the presence of these antibiotics. The *mecA* gene is located within a mobile genetic element (MGE) called the Staphylococcal Cassette Chromosome *mec* (SCC*mec*). Increasing incidences of MRSA led to the use of vancomycin to treat *S. aureus* infections. Unfortunately, this was soon followed by the emergence of vancomycin resistant strains, which were first reported in 1997 (Hiramatsu et al, 1997). Nowadays, most clinical *S*. *aureus* isolates are penicillin resistant and, on a global scale, almost half of all *S*. *aureus* infections are caused by MRSA. To make the situation worse, this opportunistic pathogen has gained resistance against all antibiotics developed over the past decades, including last resort antibiotics like linezolid and daptomycin (Stegani et al, 2010; Sabat et al, 2018).

S. aureus can acquire resistance to antibiotics by several processes. One is via mutations in the core genome, as in the case of resistance to high concentrations of ciprofloxacin. This occurs by mutation in the *gyrA* gene, which encodes DNA gyrase. Such mutations hinder the binding of ciprofloxacin to the target and, hence, the bacterium becomes resistant to this antibiotic (Campion et al, 2004). A second main mechanism of developing resistance involves horizontal gene transfer (HGT). In this case, the *S*. *aureus* bacterium acquires exogenous resistance genes carried by plasmids or other mobile genetic elements. It is believed that both the resistance to penicillin by acquisition of a *blaZ*-carrying plasmid and resistance to methicillin by acquisition of *mecA* embedded within the SCC*mec* occurred via HGT. A third phenomenon, which is sparsely studied, concerns the adaptive resistance to antibiotics. This resistance mechanism has a transient nature and occurs via changes in gene expression rather than mutation of the genome. It is believed that *S*. *aureus* employs mechanisms of adaptive resistance to manage the stresses caused by low

concentrations of antibiotics in its environment. A special form of adaptive resistance is referred to as bacterial persistence. In this phenomenon a sub-population of the bacterial population may become resistant to antibiotics without acquiring any resistance-conferring genetic changes (Cohen et al, 2013). Such persister cells have usually tuned down their metabolic activity and entered a non-dividing state. This decreases their susceptibility to the majority of antibiotics and helps the bacteria to evade the human immune defences during infection and especially upon invasion of host cells (Richards et al, 2015; Palma Median et al, 2019). Of note, intracellular growth of *S. aureus* is often accompanied by the emergence of persisters that do display genetic changes, as underlined by so-called small colony variants (Wertheim et al, 2005). In addition to the above-mentioned methods, *S. aureus* can become resistant to antibiotics through a fourth major mechanism, which involves the formation of biofilms. A biofilm is a diverse community of micro-organisms that are attached to a biotic or abiotic surface, where they grow on top of each other and become embedded in a thick matrix of extracellular polymeric substance (EPS). The EPS protects the bacteria against the host's immune system as well as antibiotics. It is usually composed of products from both the bacteria and the host, including polysaccharides, proteins and DNA. The micro-organisms in a biofilm exhibit an altered phenotype with regard to growth, gene expression and protein production, similar to the afore-mentioned persisters (Donlan et al, 2002; Archer et al, 2011). The formation of *S. aureus* biofilms on implanted biomaterials is a dreaded clinical complication due to their high resistance to antibiotics (Cohen et al, 2013; Zoller et al, 2019).

The latter part of this thesis explores the adaptive response of *S. aureus* to sub-inhibitory concentrations of ciprofloxacin.

Scope of the thesis

Chapter 1 of this thesis briefly outlays the basic concepts in regulation of bacterial gene expression and adaptation. It also addresses the various transcriptomic and proteomic techniques used to study bacterial adaptation along with their strengths and weaknesses. This chapter also introduces the two Gram-positive bacteria *B. subtilis* and *S. aureus* and the context in which their adaptive behavior was studied.

The research presented in **Chapter 2** addresses the adaptive behavior of *B. subtilis* upon sudden changes in salinity of the growth medium. Previously, it was reported that the *tat*-deficient *B. subtilis* struggles to grow when introduced in medium lacking NaCl. This growth defect was attributed to the failure of *tat* mutant strains to correctly translocate the heme peroxidase EfeB across the membrane. Importantly, EfeB is a subunit of the elemental iron transport system EfeUOB. At the start of the present studies, it was unclear why the failure to translocate EfeB is so detrimental in the absence of NaCl, and how a sub-population of *tat*-deficient *B. subtilis* can adapt to the medium without NaCl. Microarray analyses of the adaptive responses of *tat*-deficient *B. subtilis* revealed that the *tat* mutants, when introduced into medium lacking NaCl, experience severe oxidative stress and starvation, causing death of a significant portion of the bacterial population. The microarray analyses also hinted that the starving *tat* mutants were catabolizing the intracellular arginine pool as a source of energy. Upon recovery from this condition, the adapted bacteria highly expressed genes for arginine synthesis and uptake. The results described in this chapter thus focus attention on the crucial role of arginine metabolism in the adaptation to the stresses imposed on *B. subtilis* when this bacterium introduced into a medium without NaCl.

Chapter 3 describes the role of the small regulatory RNA S313 in adapting to medium lacking NaCl. S313-deficient *B. subtilis* cells showed similar growth defects as *tat*-deficient *B. subtilis*, but less severe, when introduced into medium lacking NaCl. Bioinformatic predictions and the growth defect of the S313 mutant in medium lacking NaCl hinted that S313 most likely targets the *efeUOB* operon. The subsequent experiments established that S313 actually targets *efeU* and stabilizes the *efeU* mRNA transcript, hence playing an important role in ensuring that there is a functional EfeUOB. In this way, S313 expression supports the healthy growth of *B. subtilis* in medium lacking NaCl. Additionally, microarray-based transcriptomic analyses revealed that S313 has other regulatory functions as well. In particular, the studies show that S313-deficiency results in global changes in the bacterial metabolism and a decreased resistance to antibiotics.

Considering the importance of the Tat translocase in translocation of fully folded proteins, the effects of TatAyCy overexpression were investigated using $^{14}N/^{15}N$ metabolic labelling as described in **chapter 4**. This enabled a quantitative analysis of changes occurring in the entire proteome. In particular, Tat

overexpression resulted in an extended vegetative state, which had important consequential effects on various cellular processes, including genetic competence, motility, chemotaxis and biofilm formation. Like the studies presented in **chapters 2** and **3**, the studies in **chapter 4** high-lighted the key role of arginine metabolism in the cellular adaptation to challenges imposed on the *Bacillus* cell due to altered expression of TatAyCy.

Chapter 5 redirects the focus towards adaptive responses in the pathogen *S. aureus*. In particular, the aim of the described experiments was to identify gene regulatory events that occur upon subinhibitory exposure of two closely related clinical isolates of the USA300 lineage to ciprofloxacin. One isolate showed up to 4-fold increase in the minimum inhibitory concentration (MIC) after subinhibitory ciprofloxacin exposure, whereas another isolate showed up to 8-fold increase in the MIC. RNA sequencing of the ciprofloxacin-adapted bacteria showed generally similar responses in both isolates. This included SOS and DNA repair responses, along with upregulated transcripts encoding metabolic enzymes, such as a transketolase and an aminopeptidase. Importantly, only one isolate showed induction of phages, whereas there was no evidence for phage induction in the other strain. This differential phage induction sufficient explains differences in the adaptive ciprofloxacin resistance displayed by the two isolates. Here, phage induction is associated with lower capacity to adapt to the presence of ciprofloxacin at subinhibitory levels.

Finally, **chapter 6** summarizes and discusses the major findings described in this thesis and presents an outlook for future investigations.

References

Abraham, E. P., & Chain, E. (1940). An Enzyme from Bacteria able to Destroy Penicillin. *Nature*, *146*(3713), 837.

Adams, P. P., & Storz, G. (2020). Prevalence of small base-pairing RNAs derived from diverse genomic loci. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1863*(7), 194524.

Aebersold, R., & Mann, M. (2003). Mass spectrometry-based proteomics. *Nature*, *422*(6928), 198–207.

Alwine, J. C., Kemp, D. J., & Stark, G. R. (1977). Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proceedings of the National Academy of Sciences*, *74*(12), 5350–5354.

Anderson, N. L., & Anderson, N. G. (1998). Proteome and proteomics: New technologies, new concepts, and new words. *Electrophoresis*, *19*(11), 1853–1861.

Antelo-Varela, M., Aguilar Suárez, R., Bartel, J., Bernal-Cabas, M., Stobernack, T., Sura, T., … Becher, D. (2020). Membrane Modulation of Super-Secreting "midiBacillus" Expressing the Major *Staphylococcus aureus* Antigen – A Mass-Spectrometry-Based Absolute Quantification Approach. *Frontiers in Bioengineering and Biotechnology*, *8*, NA.

Antelo-Varela, M., Bartel, J., Quesada-Ganuza, A., Appel, K., Bernal-Cabas, M., Sura, T., … Becher, D. (2019). Ariadne's Thread in the Analytical Labyrinth of Membrane Proteins: Integration of Targeted and Shotgun Proteomics for Global Absolute Quantification of Membrane Proteins. *Analytical Chemistry*, *91*(18), 11972–11980.

Archer, N. K., Mazaitis, M. J., Costerton, J. W., Leid, J. G., Powers, M. E., & Shirtliff, M. E. (2011). *Staphylococcus aureus* biofilms. *Virulence*, *2*(5), 445–459.

Barber, M. (1947). *Staphylococcal* Infection due to Penicillin-resistant Strains. *BMJ*, *2*(4534), 863–865.

Barbulovic-Nad, I., Lucente, M., Sun, Y., Zhang, M., Wheeler, A. R., & Bussmann, M. (2006). Bio-Microarray Fabrication Techniques—A Review. *Critical Reviews in Biotechnology*, *26*(4), 237–259.

Barrera, N. P., & Robinson, C. V. (2011). Advances in the Mass Spectrometry of Membrane Proteins: From Individual Proteins to Intact Complexes. *Annual Review of Biochemistry*, *80*(1), 247–271.

Beck, D., Vasisht, N., Baglieri, J., Monteferrante, C. G., van Dijl, J. M., Robinson, C., & Smith, C. J. (2013). Ultrastructural characterisation of *Bacillus subtilis* TatA complexes suggests they are too small to form homooligomeric translocation pores. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1833*(8), 1811–1819.

Becker-André, M., & Hahlbrock, K. (1989). Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY). *Nucleic Acids Research*, *17*(22), 9437–9446.

Bernal-Cabas, M., Miethke, M., Antelo-Varela, M., Aguilar Suárez, R., Neef, J., Schön, L., … van Dijl, J. M. (2020). Functional association of the stress-responsive LiaH protein and the minimal TatAyCy protein translocase in *Bacillus subtilis*. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1867*(8), 118719.

Blackstock, W. P., & Weir, M. P. (1999). Proteomics: quantitative and physical mapping of cellular

proteins. *Trends in Biotechnology*, *17*(3), 121–127.

Blyn, L. B., Braaten, B. A., & Low, D. A. (1990). Regulation of pap pilin phase variation by a mechanism involving differential dam methylation states. *The EMBO Journal*, *9*(12), 4045–4054.

Campion, J. J., McNamara, P. J., & Evans, M. E. (2004). Evolution of Ciprofloxacin-Resistant *Staphylococcus aureus* in In Vitro Pharmacokinetic Environments. *Antimicrobial Agents and Chemotherapy*, *48*(12), 4733–4744.

Carrier, M.-C., Lalaouna, D., & Massé, E. (2018). Broadening the Definition of Bacterial Small RNAs: Characteristics and Mechanisms of Action. *Annual Review of Microbiology*, *72*(1), 141–161.

Casadesús, J., & Low, D. (2006). Epigenetic Gene Regulation in the Bacterial World. *Microbiology and Molecular Biology Reviews*, *70*(3), 830–856.

Cech, T. R., & Steitz, J. A. (2014). The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. *Cell*, *157*(1), 77–94.

Chareyre, S., & Mandin, P. (2018). Bacterial Iron Homeostasis Regulation by sRNAs. *Microbiology Spectrum*, *6*(2).

Chu, Y., & Corey, D. R. (2012). RNA Sequencing: Platform Selection, Experimental Design, and Data Interpretation. *Nucleic Acid Therapeutics*, *22*(4), 271–274.

Cohen, N. R., Lobritz, M. A., & Collins, J. J. (2013). Microbial Persistence and the Road to Drug Resistance. *Cell Host & Microbe*, *13*(6), 632–642.

Cox, J., & Mann, M. (2011). Quantitative, High-Resolution Proteomics for Data-Driven Systems Biology. *Annual Review of Biochemistry*, *80*(1), 273–299.

Dalbey, R. E., Wang, P., & van Dijl, J. M. (2012). Membrane Proteases in the Bacterial Protein Secretion and Quality Control Pathway. *Microbiology and Molecular Biology Reviews*, *76*(2), 311–330.

Darwin, Charles (1859), On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life (Full image view 1st ed.), London: John Murray, p. 502.

Dodd, M. S., Papineau, D., Grenne, T., Slack, J. F., Rittner, M., Pirajno, F., … Little, C. T. S. (2017). Evidence for early life in Earth's oldest hydrothermal vent precipitates. *Nature*, *543*(7643), 60–64.

Donlan, R. M., & Costerton, J. W. (2002). Biofilms: Survival Mechanisms of Clinically Relevant Microorganisms. *Clinical Microbiology Reviews*, *15*(2), 167–193.

Driessen, A. J. M., & Nouwen, N. (2008). Protein Translocation Across the Bacterial Cytoplasmic Membrane. *Annual Review of Biochemistry*, *77*(1), 643–667.

Eder, S., Shi, L., Jensen, K., Yamane, K., & Hulett, F. M. (1996). A *Bacillus subtilis* secreted phosphodiesterase/alkaline phosphatase is the product of a Pho regulon gene, phoD. *Microbiology*, *142*(8), 2041–2047.

Forbes, S., Dobson, C. B., Humphreys, G. J., & McBain, A. J. (2014). Transient and Sustained Bacterial Adaptation following Repeated Sublethal Exposure to Microbicides and a Novel Human Antimicrobial Peptide. *Antimicrobial Agents and Chemotherapy*, *58*(10), 5809–5817.

Frain, K. M., Robinson, C., & van Dijl, J. M. (2019). Transport of Folded Proteins by the Tat System. *The Protein Journal*, *38*(4), 377–388.

Forde, B. M., & O'Toole, P. W. (2013). Next-generation sequencing technologies and their impact on microbial genomics. Briefings in Functional Genomics, 12(5), 440–453.

Fröbel, J., Rose, P., Lausberg, F., Blümmel, A.-S., Freudl, R., & Müller, M. (2012). Transmembrane insertion of twin-arginine signal peptides is driven by TatC and regulated by TatB. *Nature Communications*, *3*(1), NA.

Goosens, V. J., De-San-Eustaquio-Campillo, A., Carballido-López, R., & van Dijl, J. M. (2015). A Tat ménage à trois — The role of *Bacillus subtilis* TatAc in twin-arginine protein translocation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1853*(10), 2745–2753.

Goosens, V. J., Monteferrante, C. G., & van Dijl, J. M. (2014). The Tat system of Gram-positive bacteria. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1843*(8), 1698–1706.

Goosens, V. J., Otto, A., Glasner, C., Monteferrante, C. C., van der Ploeg, R., Hecker, M., … van Dijl, J. M. (2013). Novel Twin-Arginine Translocation Pathway-Dependent Phenotypes of *Bacillus subtilis* Unveiled by Quantitative Proteomics. *Journal of Proteome Research*, *12*(2), 796–807.

Goosens, V. J., & van Dijl, J. M. (2016). Twin-Arginine Protein Translocation. *Current Topics in Microbiology and Immunology*, 69–94.

Güell, M., Yus, E., Lluch-Senar, M., & Serrano, L. (2011). Bacterial transcriptomics: what is beyond the RNA horiz-ome? *Nature Reviews Microbiology*, *9*(9), 658–669.

Hackbarth, C. J., & Chambers, H. F. (1993). blaI and blaR1 regulate beta-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, *37*(5), 1144–1149.

Harwood, C. R., & Cranenburgh, R. (2008). *Bacillus* protein secretion: an unfolding story. *Trends in Microbiology*, *16*(2), 73–79.

Hashimshony, T., Wagner, F., Sher, N., & Yanai, I. (2012). CEL-Seq: Single-Cell RNA-Seq by Multiplexed Linear Amplification. *Cell Reports*, *2*(3), 666–673.

Haugen, S. P., Ross, W., & Gourse, R. L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. *Nature Reviews Microbiology*, *6*(7), 507–519.

Heller, M. J. (2002). DNA Microarray Technology: Devices, Systems, and Applications. *Annual Review of Biomedical Engineering*, *4*(1), 129–153.

Hiramatsu, K., Aritaka, N., Hanaki, H., Kawasaki, S., Hosoda, Y., Hori, S., … Kobayashi, I. (1997). Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. *The Lancet*, *350*(9092), 1670–1673.

Hou, B., Heidrich, E. S., Mehner-Breitfeld, D., & Brüser, T. (2018). The TatA component of the twin-arginine translocation system locally weakens the cytoplasmic membrane of *Escherichia coli* upon protein substrate binding. *Journal of Biological Chemistry*, *293*(20), 7592–7605.

Jevons, M. P. (1961). "Celbenin" - resistant *Staphylococci*. *BMJ*, *1*(5219), 124–125.

Jongbloed, J. D. H., Grieger, U., Antelmann, H., Hecker, M., Nijland, R., Bron, S., & Van Dijl, J. M. (2004). Two minimal Tat translocases in *Bacillus*. *Molecular Microbiology*, *54*(5), 1319–1325.

Jongbloed, J. D. H., Martin, U., Antelmann, H., Hecker, M., Tjalsma, H., Venema, G., … Müller, J. (2000). TatC Is a Specificity Determinant for Protein Secretion via the Twin-arginine Translocation Pathway. *Journal of Biological Chemistry*, *275*(52), 41350–41357.

Jongbloed, J. D. H., van der Ploeg, R., & van Dijl, J. M. (2006). Bifunctional TatA subunits in minimal Tat protein translocases. *Trends in Microbiology*, *14*(1), 2–4.

Jørgensen, M. G., Pettersen, J. S., & Kallipolitis, B. H. (2020). sRNA-mediated control in bacteria: An increasing diversity of regulatory mechanisms. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1863*(5), 194504.

Krishnappa, L., Dreisbach, A., Otto, A., Goosens, V. J., Cranenburgh, R. M., Harwood, C. R., … van Dijl, J. M. (2013). Extracytoplasmic Proteases Determining the Cleavage and Release of Secreted Proteins, Lipoproteins, and Membrane Proteins in *Bacillus subtilis*. *Journal of Proteome Research*, *12*(9), 4101–4110.

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., … Chambers, H. F. (2011). Clinical Practice Guidelines by the Infectious Diseases Society of America for the Treatment of Methicillin-Resistant *Staphylococcus aureus* Infections in Adults and Children. *Clinical Infectious Diseases*, *52*(3), e18–e55.

Lüke, I., Handford, J. I., Palmer, T., & Sargent, F. (2009). Proteolytic processing of *Escherichia coli* twin-arginine signal peptides by LepB. *Archives of Microbiology*, *191*(12), 919–925.

McDaniel, B. A. M., Grundy, F. J., Artsimovitch, I., & Henkin, T. M. (2003). Transcription termination control of the S box system: Direct measurement of S-adenosylmethionine by the leader RNA. *Proceedings of the National Academy of Sciences*, *100*(6), 3083–3088.

McGettigan, P. A. (2013). Transcriptomics in the RNA-seq era. *Current Opinion in Chemical Biology*, *17*(1), 4–11.

Miethke, M., Monteferrante, C. G., Marahiel, M. A., & van Dijl, J. M. (2013). The *Bacillus subtilis* EfeUOB transporter is essential for high-affinity acquisition of ferrous and ferric iron. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1833*(10), 2267–2278.

Monteferrante, C. G., Miethke, M., van der Ploeg, R., Glasner, C., & van Dijl, J. M. (2012). Specific Targeting of the Metallophosphoesterase YkuE to the *Bacillus* Cell Wall Requires the Twin-arginine Translocation System. *Journal of Biological Chemistry*, *287*(35), 29789–29800.

Mould, R. M., & Robinson, C. (1991). A proton gradient is required for the transport of two lumenal oxygen-evolving proteins across the thylakoid membrane. *J Biol Chem*, *266*(19), 12189–12193

Myers, C. L., Li, F. K. K., Koo, B.-M., El-Halfawy, O. M., French, S., Gross, C. A., … Brown, E. D. (2016). Identification of Two Phosphate Starvation-induced Wall Teichoic Acid Hydrolases Provides First Insights into the Degradative Pathway of a Key Bacterial Cell Wall Component. *Journal of Biological Chemistry*, *291*(50), 26066–26082.

Nicolas, P., Mader, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., … Noirot, P. (2012). Condition-Dependent Transcriptome Reveals High-Level Regulatory Architecture in *Bacillus subtilis*. *Science*, *335*(6072), 1103–1106.

Ozsolak, F., & Milos, P. M. (2010). RNA sequencing: advances, challenges and opportunities. *Nature Reviews Genetics*, *12*(2), 87–98.

Palma Medina, L. M., Becker, A.-K., Michalik, S., Yedavally, H., Raineri, E. J. M., Hildebrandt, P., … Völker, U. (2019). Metabolic Cross-talk Between Human Bronchial Epithelial Cells and Internalized *Staphylococcus aureus* as a Driver for Infection. *Molecular & Cellular Proteomics*, *18*(5), 892–908.

Palmer, T., & Berks, B. C. (2012). The twin-arginine translocation (Tat) protein export pathway. *Nature Reviews Microbiology*, *10*(7), 483–496.

Palmer, T., Sargent, F., & Berks, B. C. (2005). Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends in Microbiology*, *13*(4), 175–180.

Park, E., & Rapoport, T. A. (2012). Mechanisms of Sec61/SecY-Mediated Protein Translocation Across Membranes. *Annual Review of Biophysics*, *41*(1), 21–40.

Petersohn, A., Antelmann, H., Gerth, U., & Hecker, M. (1999). Identification and transcriptional analysis of new members of the σB regulon in *Bacillus subtilis*. *Microbiology*, *145*(4), 869–880.

Perkins, J. B., Bower, S., Howitt, C. L., Yocum, R. R., & Pero, J. (1996). Identification and characterization of transcripts from the biotin biosynthetic operon of *Bacillus subtilis*. *Journal of Bacteriology*, *178*(21), 6361–6365.

Richards, R. L., Haigh, R. D., Pascoe, B., Sheppard, S. K., Price, F., Jenkins, D., … Morrissey, J. A. (2015). Persistent *Staphylococcus aureus* Isolates from Two Independent Cases of Bacteremia Display Increased Bacterial Fitness and Novel Immune Evasion Phenotypes. *Infection and Immunity*, *83*(8), 3311–3324.

Romanov, V., Davidoff, S. N., Miles, A. R., Grainger, D. W., Gale, B. K., & Brooks, B. D. (2014). A critical comparison of protein microarray fabrication technologies. *The Analyst*, *139*(6), 1303–1326.

Sabat, A. J., Tinelli, M., Grundmann, H., Akkerboom, V., Monaco, M., Del Grosso, M., … Friedrich, A. W. (2018). Daptomycin Resistant *Staphylococcus aureus* Clinical Strain With Novel Non-synonymous Mutations in the mprF and vraS Genes: A New Insight Into Daptomycin Resistance. *Frontiers in Microbiology*, *9*, NA.

Schäfer, K., Künzler, P., Schneider, K., Klingl, A., Eubel, H., & Carrie, C. (2020). The Plant Mitochondrial TAT Pathway Is Essential for Complex III Biogenesis. *Current Biology*, *30*(5), 840-853.e5.

Sohanpal, B. K., El-Labany, S., Lahooti, M., Plumbridge, J. A., & Blomfield, I. C. (2004). Integrated regulatory responses of fimB to N-acetylneuraminic (sialic) acid and GlcNAc in *Escherichia coli* K-12. *Proceedings of the National Academy of Sciences*, *101*(46), 16322–16327.

Stefani, S., Bongiorno, D., Mongelli, G., & Campanile, F. (2010). Linezolid Resistance in *Staphylococci*. *Pharmaceuticals*, *3*(7), 1988–2006.

Storz, G., Vogel, J., & Wassarman, K. M. (2011). Regulation by Small RNAs in Bacteria: Expanding Frontiers. *Molecular Cell*, *43*(6), 880–891.

Svensson, V., Vento-Tormo, R., & Teichmann, S. A. (2018). Exponential scaling of single-cell RNA-seq in the past decade. *Nature Protocols*, *13*(4), 599–604.

Tjalsma, H., Antelmann, H., Jongbloed, J. D. H., Braun, P. G., Darmon, E., Dorenbos, R., … van Dijl, J. M. (2004). Proteomics of Protein Secretion by *Bacillus subtilis*: Separating the "Secrets" of the Secretome. *Microbiology and Molecular Biology Reviews*, *68*(2), 207–233.

van Dijl, J. M., & Hecker, M. (2013). *Bacillus subtilis*: from soil bacterium to super-secreting cell factory. *Microbial Cell Factories*, *12*(1), 3.

Wang, K. C., & Chang, H. Y. (2011). Molecular Mechanisms of Long Noncoding RNAs. *Molecular Cell*, *43*(6), 904–914.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, *10*(1), 57–63.

Wertheim, H. F. L., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A., & Nouwen, J. L. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. *The Lancet Infectious Diseases*, *5*(12), 751–762.

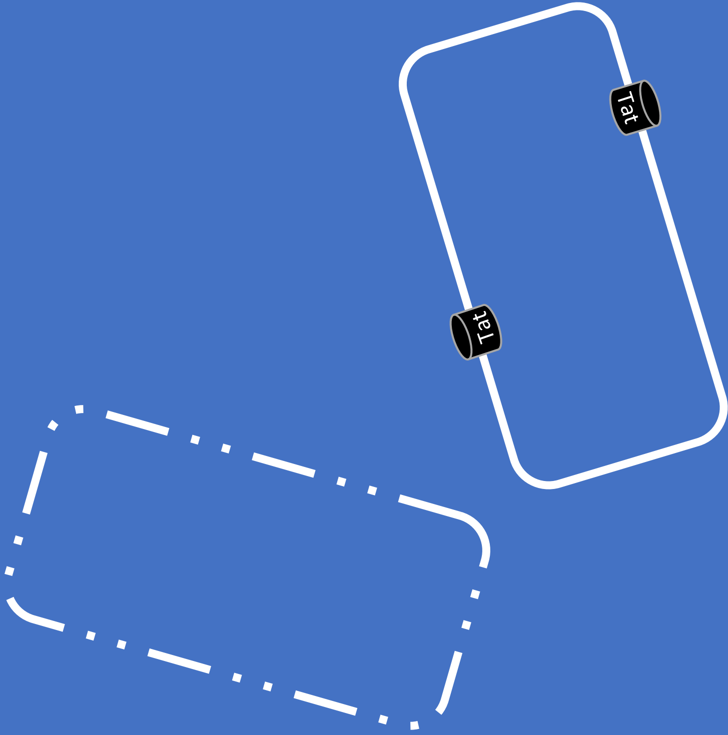
Weston, A. D., & Hood, L. (2004). Systems Biology, Proteomics, and the Future of Health Care: Toward Predictive, Preventative, and Personalized Medicine. *Journal of Proteome Research*, *3*(2), 179–196.

Velculescu, V. E., Zhang, L., Vogelstein, B., & Kinzler, K. W. (1995). Serial Analysis of Gene Expression. *Science*, *270*(5235), 484–487.

Yen, M.-R., Tseng, Y.-H., Nguyen, E. H., Wu, L.-F., & Saier, M. H. (2002). Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Archives of Microbiology*, *177*(6), 441–450.

Yuan, J., Zweers, J. C., van Dijl, J. M., & Dalbey, R. E. (2009). Protein transport across and into cell membranes in bacteria and archaea. *Cellular and Molecular Life Sciences*, *67*(2), 179–199.

Zoller, S. D., Park, H. Y., Olafsen, T., Zamilpa, C., Burke, Z. D. C., Blumstein, G., … Bernthal, N. M. (2019). Multimodal imaging guides surgical management in a preclinical spinal implant infection model. *JCI Insight*, *4*(3), NA.


Tat
Tat