Engineering approaches to investigate pneumococcal gene expression regulation and antibiotic resistance development
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Chapter 1

Thesis Introduction

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The pneumococcus: a global health threat

*Streptococcus pneumoniae*, the pneumococcus, is a Gram-positive bacterium that is commonly found in the nasopharynx of children, and furthermore in approximately one out of ten adults. The organism is part of the commensal microbiota of the human upper respiratory tract together with non-pathogenic bacteria, such as the closely related *Streptococcus mitis*, and with bacteria that frequently cause disease, such as *Moraxella catarrhalis*, *Haemophilus influenzae*, *Neisseria meningitides* and *Staphylococcus aureus*. A study in the Netherlands found that pneumococcal colonization peaks at the age of 3 years, when 55% of children are carriers, and thereafter steadily decreases until the age of 10 years, when a plateau of 8% is reached. The likelihood of being a carrier, however, is strongly influenced by the social environment of individuals; children attending day-care centers are at least one and a half times as likely to be colonized compared with children that are cared for at home.

Next to an innocuous prevalence in the human nasopharynx, *S. pneumoniae* can cause mucosal infections, such as otitis media and pneumonia, and invasive infections, such as meningitis and sepsis. Pneumococcal disease mostly affects young children and elderly people, and furthermore people suffering from immunodeficiency. The age distribution of patients with community-acquired pneumonia, for example, is U-shaped (estimated between 1.5 and 14.0 cases per 1000 persons per year, in dependency of the region and population characteristics), mostly occurring in children younger than 5 years and adults older than 65 years. *S. pneumoniae* is the main cause of community-acquired pneumonia, responsible for 35% of incidences in Europe and 27.3% of incidences worldwide. Because of the high rate of both morbidity and mortality, *S. pneumoniae* is considered the biggest bacterial health threat worldwide, accounting for more than one million deaths per year.

Historically, *S. pneumoniae* was first isolated by Louis Pasteur and George Miller Sternberg in 1881, and the occurrence of the organism in patients with pneumonia resulted in the naming “pneumococcus”. In 1974, the Latin name “*Streptococcus pneumoniae*” was
chosen because of the characteristic of the bacterium to form chains in liquid culture. For the development of the research field of molecular genetics, *S. pneumoniae* played a key role when Frederick Griffith used the organism in 1928 to demonstrate that bacteria are able to transfer genetic information. Griffith showed that a non-invasive pneumococcal strain, forming rough colonies, could colonize and kill mice when co-providing material of a heat-inactivated invasive strain, forming smooth colonies\(^\text{10}\). In 1944, Oswald Avery and colleagues demonstrated that the material responsible for genetic transformation is DNA, and thus that DNA of invasive strains was the carrier of the information that enabled previously non-invasive pneumococci to infect mice\(^\text{11}\).

**Genome and evolution of *S. pneumoniae***

A major contributing factor that enables *S. pneumoniae* to colonize the human host is the expression of an extracellular polysaccharide capsule. This capsule protects the pneumococcal cell wall from recognition by complement and antibody opsonins and therefore prevents from phagocytosis\(^\text{12}\). More than 90 different capsules are known to date referred to as serotypes\(^\text{13,14}\). The corresponding number of different capsule operons reflects the broad pan-genome of *S. pneumoniae*, and thus the high degree of genomic plasticity within the species. Pneumococcus harbors a small genome of approximately 2.1 Mb that contains more than 2000 predicted protein coding regions\(^\text{15}\); however, the core genome comprises only 1647 coding regions, and the relatively large size of the remaining accessory genome leads to an average sequence identity of merely 74% between individual strains at the nucleotide level\(^\text{16}\).

Genomic variance within *S. pneumoniae* is supported by natural competence, a process involving more than 100 genes\(^\text{17}\) that allows for the uptake of exogenous DNA and integration into the genome via homologous recombination. Frequently occurring opportunities for horizontal gene transfer enable pneumococci to quickly adapt to changing environmental conditions by recombining favorable genetic traits. It is speculated that strong intraspecies competition was the driving force towards the evolution of natural competence\(^\text{18}\). Pneumococci express bacteriocins from the highly heterologous *blp* locus which inhibit strains that do not express a cognate immunity factor, and the ability to become competent consequently provided a chance to rapidly acquire immunity genes\(^\text{19}\). Alternatively, competence might represent an adaptation to the high abundance of oxygen in the nasopharynx, allowing for straightforward repair of oxidative DNA damage\(^\text{19}\). Recently, it has been hypothesized that competence also acts as an effective defense against mobile genetic elements\(^\text{20}\).
In the attempt of following and understanding the epidemiology of *S. pneumoniae*, several systems were developed to categorize individual strains. The first system consisted of immunological typing based on the serotype. Quellung reaction, a method described by Fred Neufeld in 1902, uses antibodies that attach to capsular antigens and thereby alter both the microscopic appearance (capsule swelling) and the macroscopic appearance (cell agglutination) of pneumococci. The main limitation of this approach relies in a unique focus on capsule genes, which can be readily replaced in a process referred to as capsule switching. More recent approaches involve genomic sequencing, such as the sequencing of seven housekeeping genes called multilocus sequence typing (MLST). The combination of these sequences provides an allelic profile called the sequence type (ST), and STs are grouped into clonal complexes (CC) that typically contain matching alleles in five or six out of the seven loci. Importantly, STs (or CCs) are better indicators for the genetic relatedness between strains, as compared with serotypes. In 1997, the Pneumococcal Molecular Epidemiology Network (PMEN) was founded with the aim of monitoring the increasing spread of antibiotic-resistant pneumococcal strains. A nomenclature was developed that integrates the above-mentioned systems, for instance strain Spain, referring to the country of origin, the serotype, and a consecutive clone number, as determined by MLST.
Colonization, pathogenesis and clearance

*S. pneumoniae* spreads from person to person via respiratory droplets, for example when an in general healthy carrier exhales, coughs or sneezes in the proximity of others. For this reason increased crowding, such as in hospitals, schools or day-care centers, results in higher rates of pneumococcal propagation. Importantly, pneumococcal disease does not start directly from contact with contaminated secretions but only develops subsequent to colonization of the upper respiratory tract. It is also worth noting that exclusively nasopharyngeal carriage, but none of the diseases caused by *S. pneumoniae*, promotes the dissemination of the organism; sick carriers, in contrast, limit pneumococcal spreading because patients reduce their social interactions.

Pneumococcal colonization of the upper respiratory tract is a dynamic process that involves competition with the resident microbiota and challenges by the mucosal immune response. After transmission, *S. pneumoniae* transits to the ventral nasal space and attaches to the epithelial lining via surface-proteins that are anchored to the pneumococcal cell wall. Upon contact with the epithelium, an inflammatory response is triggered: the epithelial barrier opens at cellular junctions allowing for the influx of inflammatory cells, and the complement cascade and cytokine production become activated. Within one day, a mild rhinitis (runny nose) develops and neutrophils associated with dense collections of pneumococci can be observed. However, the impact of this initial inflammatory response was found to affect pneumococcal colonization only to a minimal extent. There is evidence that triggering an inflammation even provides an advantage for *S. pneumoniae*, such as the clearance of niche competitors or increased host to host spreading via the stimulation of secretion production, outweighing the negative consequences of an activated immune system.

The period from initial settlement to clearance of pneumococci usually spans several weeks. The progression of nasopharyngeal colonization is characterized by the absence of an overt pro-inflammatory response, and immune-quiescent *S. pneumoniae* were shown to organize in the form of biofilms. Clearance eventually involves the activation of the classical complement pathway, production of type-specific antibodies (targeting capsule polysaccharides) and the recruitment of macrophages. Pneumococcal colonization of the upper respiratory tract is mostly asymptomatic, and carriers are usually not aware of being infected. In the case of secondary infection, or vaccination against the homologous serotype,
pneumococcal clearance proceeds more rapidly because type-specific antibodies mediate a direct effective opsonophagocytosis by neutrophils during the initial mucosal inflammatory response.

The route towards pneumococcal disease, or in other words the invasion of sterile tissue starting from the nasopharynx, is less well understood and probably involves multifactorial processes. Local dispersal presumably gives access to proximal mucosal tissues, such as the middle ear and the sinus, breaching the epithelial barrier is believed to enable access to the blood and via the bloodstream also to the meninges, and both aspiration and the blood-borne route may provide access to the lung. It is generally accepted that the lack of a brisk mucosal inflammatory response, and thus failure to contain the initial infection of the upper airway, increases the risk of a subsequent systemic infection, such as the life-threatening acute pneumonia or meningitis. Prevention of nasopharyngeal colonization, which is not only the starting point for disease but also the basis for horizontal spread, is consequently key for managing the global health burden imposed by *S. pneumoniae*.

**Figure 2 | Pathogenic route for *S. pneumoniae* infection.** Adapted from Bogaert et al., 2004.
Pneumococcal virulence factors

The pneumococcal genome encodes virulence factors, which are genes of special function that enable human colonization and that provide protection against attacks of the human immune system. A comprehensive list can be found elsewhere\textsuperscript{43–45}. The most important virulence factor is the above-mentioned pneumococcal capsule (\textit{cps}), expressed in form of a variety of structurally and immunologically unique extracellular polysaccharides that prevent from phagocytosis. The prevalence of individual serotypes in carriage and invasive disease was found to be unequally distributed, indicating that different capsules provide varying protection\textsuperscript{46,47}. The potential of causing disease, however, is not only dependent on the serotype but also involves other strain-specific characteristics\textsuperscript{48}. Interestingly, there is evidence that serotypes showing high carriage frequency are less invasive, supporting the hypothesis that causing disease represents a disadvantage for the dissemination of a pneumococcal strain\textsuperscript{19,48}.

\textit{S. pneumoniae} expresses a single toxin, pneumolysin (\textit{ply}), a pore-forming enzyme that is toxic for a wide variety of host cells and that triggers strong inflammatory responses\textsuperscript{49}. Pneumolysin promotes pneumococcal colonization and disease via several modes of action; for example, its cytotoxic effect on epithelial cells slows ciliary beating and thus reduces mucus and bacterial clearance\textsuperscript{50,51}. During systemic infections, the majority of pneumococcus-induced tissue damaging is attributed to the activity of pneumolysin\textsuperscript{49}. Furthermore, the structural homology of pneumolysin to the immunoglobulin G (IgG) Fc region was shown to result in a depletion of serum complement at the center of inflammation, which in turn reduces opsonophagocytosis\textsuperscript{52,53}. Another mechanism how \textit{S. pneumoniae} impairs opsonins was found in the sequential activities of exoglycosidases (\textit{nanaA}, \textit{bgaA} and \textit{strH}), which target, amongst others, human secretory immunoglobulin A (IgA) and C-reactive protein (CRP)\textsuperscript{54}. IgA, which is the principal antibody isotype of mucosal surfaces, is furthermore targeted by a pneumococcal protease (\textit{iga}) that cleaves off Fab fragments; these fragments mask pneumococcal surface antigens from functional antibodies and thus prevent from agglutination and host recognition\textsuperscript{55}. All of the described virulence factors activities could be demonstrated to play important roles during nasopharyngeal colonization, and they consequently all contribute to a successful commensal lifestyle. Nevertheless, virulence factors also enable invasive infections, and a deeper understanding of the selection pressure towards virulence factor acquisition is required to improve our ability of containing pneumococcal pathogenicity.
Vaccination, antibiotic therapy and resistance

The most important strategy to contain pneumococcal disease on the society level is large-scale vaccination. Capsule polysaccharides are the principal immunogen of *S. pneumoniae*, and they consequently represent the primary target for vaccination. Pneumococcal polysaccharide vaccine (PPSV) was first described in 1945 and initially provided protection against a limited number of 4 serotypes. PPSV only became effective and widely distributed in 1970 when a 14-valent vaccine was developed. PPSV induces B cell-dependent immune response characterized by the production of IgM, and protection lasts for approximately 5 years. The currently available PPSV, Pneumovax 23, targets the 23 most prevalent serotypes causing invasive disease, covering 85-90% of all cases. While PPSV protects from invasive disease, it does not reduce carriage rate, and furthermore fails to mount an adequate immunity in infants and elderly people, the major risk groups of pneumococcal disease.

To overcome the limitations of PPSV, a second vaccine type was developed and reported in 1991. Pneumococcal conjugate vaccine (PCV) contains capsule polysaccharides that are covalently linked to a highly immunogenic carrier protein, CRM197 – a (nontoxic) recombinant diphtheria toxin. PCV activates T cell-dependent humoral immune response characterized by isotype switching to IgG and secretory IgA, and it furthermore results in the formation of memory B cells. The currently available PCV, Prevnar 13 (PCV13, successor of the initial PCV7) was shown to provide highly effective long-term protection against pneumococcal disease. In contrast to PPSV, PCV decreases nasopharyngeal carriage rates, via IgA deposition in the mucosa, and thus also provides herd immunity for non-vaccinated individuals. Note that the Dutch national vaccination program implements a decavalent PCV.

In recent years, routine vaccination against *S. pneumoniae* was shown to be accompanied by a phenomenon called serotype replacement, the rise of non-vaccine serotypes causing mucosal infections and invasive disease. Additionally, it was shown that vaccination related reduction of pneumococcal carriage led to increased carriage of niche competitors, such as *S. aureus*. Replacement disease could, in the long run, erode the substantial benefits of pneumococcal vaccination, and regular adaptation of the PPSV and PCV formulation might be required to guarantee lasting effectiveness against the changing composition of prevailing serotypes.

Another historic milestone in combatting pneumococcal disease was the discovery of antibiotics. Starting from the late 1940s, penicillin (discovered in 1928 by Alexander
Fleming\textsuperscript{72} was commonly used to treat community-acquired pneumonia\textsuperscript{73}. However, after a first report in 1967\textsuperscript{74}, resistant strains emerged and became increasingly prevalent, compromising antibiotic therapy. Penicillin-resistance (and $\beta$-lactam drug-resistance of pneumococcus in general) is characterized by mutated penicillin binding proteins (PBPs – catalyzing the final step of peptidoglycan synthesis) with reduced drug affinity\textsuperscript{75}. Resistant strains carry mosaic $pbp$ alleles that show sequence deviations of up to 21\% compared with native alleles, indicating that resistance acquisition was likely driven by recombination events with commensal streptococci\textsuperscript{76}. Furthermore, clonal dissemination plays a major role

\begin{figure}[h]
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\caption{Antibody responses to polysaccharide antigens (A) and polysaccharide–protein conjugates (B). Adapted from Ada, 2001.}
\end{figure}
in the spreading of antibiotic resistance\textsuperscript{77}. Remarkably, unencapsulated pneumococci that are not included in vaccines show high frequencies of receipt and donation of recombined DNA fragments, and thus promote the spreading of antibiotic resistance and the adaptation of the species as a whole\textsuperscript{78}.

Resistances to all known antibiotic families have been found in \textit{S. pneumoniae} to date\textsuperscript{79}, and the first multidrug-resistant strain, defined to show resistance against three or more classes of antibiotics, was reported in 1977\textsuperscript{80}. Studies between 2003 and 2005 found that multidrug-resistant strains represented up to 90.7\% of isolates\textsuperscript{81,82}, and other studies reported a strong link between the frequency of antibiotics prescription and the rise of resistant strains\textsuperscript{83–86}. Interestingly, the introduction of PCV resulted in the containment of invasive multidrug-resistant strains, and vaccination might thus contribute to solving the issue imposed by multidrug resistance\textsuperscript{87}. Nevertheless, the genomic plasticity of \textit{S. pneumoniae} allows the organism to rapidly adapt to clinical interventions, such as antibiotic therapy, and new medications and treatment strategies will need to be developed on a regular basis to guarantee a cure for patients suffering from pneumococcal disease\textsuperscript{88}.

\textbf{Gene expression regulation}

Fast changing environments, such as the human nasopharynx, represent a challenge for their inhabitants, and bacteria are required to sense, analyze and respond to changing environmental conditions; these processes are integrated in the gene expression control. In the case of the pneumococcus, gene expression control can be observed both at the population level, such as competence development and bacteriocin production that are triggered via quorum-sensing, and at the single-cell level, such as virulence factor expression. Controlling the amount of capsule expression, for example, and thus regulating the capsule thickness, seems to be of fundamental importance as evidenced by the presence of a complex regulation mechanism. This mechanism allows for reversible genomic rearrangements, giving rise to distinct DNA methylation patterns and in turn to different gene expression profiles\textsuperscript{89}. High and low capsule expression can provide both advantages and disadvantages: thick capsules protect more efficiently from opsonophagocytosis while thin capsules enable more efficient attachment to mucosal surfaces and protection from antimicrobial peptides\textsuperscript{90}. Cells from parental \textit{S. pneumoniae} populations were shown to be able to switch between capsule expression types, resulting in distinct colony morphologies. This phenomenon is called phase variation\textsuperscript{91,92}; colonies can be distinguished as either opaque (cells with a thick capsule) or transparent (cells with a thin capsule)\textsuperscript{93}. The capsule is also controlled at the post-translational level by the autolysin LytA that sheds the capsule upon adherence to epithelial cells\textsuperscript{90}. 
Gene expression regulation, in general, enables bacteria to respond to their environment, and different regulatory networks allow for responses in distinct manners. The different requirements for gene expression regulation are intuitive to understand when comparing two examples: the expression of the tetracycline resistance gene \textit{tetA} (encoded on the Tn10 transposon) of \textit{Escherichia coli}, and the development of the motile phenotype of \textit{Bacillus subtilis}. TetA confers tetracycline resistance by exporting the antibiotic from the cytoplasm, and the expression of \textit{tetA} is optimized to follow a graded response. The Tn10 transposon encodes transcriptional regulators that are responsible for controlling an efficient expression level of the TetA; at increasing concentration of tetracycline, a corresponding increase in TetA concentration is required to facilitate more antibiotic efflux\textsuperscript{94}. Permanent high levels of TetA, however, are not desirable because of interference with the cellular metabolism and for energy budget reasons.

The regulation of genes encoding the bacterial flagellum of \textit{B. subtilis}, in contrast, requires a different response pattern. In exponentially growing \textit{B. subtilis} cultures the formation of a subpopulation of swimming cells can be observed\textsuperscript{95}. The induction of this phenotype requires a more complex regulation architecture because it represents an all-or-nothing decision. In this example, it is more difficult to determine input factors precisely, and triggering conditions depend on the interplay between a set of factors, such as nutrient availability and quorum sensing. Importantly, when these triggering conditions establish, the likelihood of switching to the motile phenotype increases for each individual cell of the population. Motile and non-motile subpopulations of \textit{B. subtilis} coexist within the same environment, and the ratio of the two cell types depends on the progression of the triggering conditions\textsuperscript{96}. The response to the environmental input is thus discontinuous; cells either do express flagellum-related genes or they do not. Such response patterns can be regulated, for example, by a genetic bistable switch.

**Bistable switches**

To understand the mode of action of bistable switches, it is best to illustrate the individual key factor activities with the help of a diagram (see Fig. 4)\textsuperscript{97}. The most reduced functional example of such a network is a positive feedback loop of a transcriptional activator that regulates its own expression (scheme in Fig. 4b). The promoter activity of such a system is required to show hypersensitivity, or in other words it requires a dose-response relationship that gives rise to a sigmoidal curve. In a system without feedback (scheme in Fig. 4a), low transcription rates are observed for low activator concentrations, and high transcription rates for high activator concentrations. Even without any activator present there is a minor
promoter activity called the leakiness. The promoter activity can be induced to a specific maximum; at this point, other factors become limiting, such as RNA polymerase. The sigmoidal expression curve, instead of a saturation curve, is caused by the cooperative binding behavior of transcription factors (red curve, Fig. 4a). The antagonistic process that limits the accumulation of proteins is the active decay by proteolysis, and the passive decay due to cell growth mediated dilution. The combination of these two processes is in the following referred to as degradation; note that, in the case of stable proteins, the dominant effect is dilution. During exponential growth, proteins show a specific half-life time in dependency of the bacterial growth rate, resulting in a linear relationship between the current protein concentration and the degradation rate (black line, Fig. 4a).

For a system with positive feedback (the activator regulates the expression of its own gene; scheme in Fig. 4b), the activator concentration within a cell settles at equilibria called

**Figure 4 | Bistable switches in open and closed loop representation.** Adapted from Siebring et al., 2012.
steady states, in which activator production equals activator degradation. These steady states can be found in the open loop representation at intersections of the sigmoidal expression curve with the degradation line (Fig. 4a). Whenever the production rate is higher than the degradation rate, the net effect will be a build-up of activator. In contrast, more degradation than production results in a net loss of activator. In the given example, the system is arranged to have three intersections, and thus three equilibria, of which only the higher and the lower ones are stable (Low state and High state, Fig. 4a). When increasing or decreasing the activator concentration, to simulate naturally occurring fluctuations, the system always falls back to these two stable states. In contrast, the central intersection is unstable (Watershed, Fig. 4a); increasing the activator concentration pulls the system towards the High state and decreasing the activator concentration pulls the system towards the Low state. This unstable steady state is called the watershed, or also referred to as the threshold of the system. Importantly, in the case of a close proximity of one of the stable state to the watershed (within the reach of the fluctuation amplitude), the system will eventually switch to the other stable state.

A more complex example of a bistable switch is the scenario of an inhibitor hampering the functionality of a transcriptional activator (scheme in Fig. 4d). In this case, without inhibition, the expression curve and the degradation line cross only once, representing the High state (orange curve in Fig. 4c). Increasing the inhibitor concentration either stretches (for low inhibitor affinity) or shifts (for high inhibitor affinity) the expression curve, as less of the abundant activator is actually functional. At high inhibitor concentrations, there is consequently also only one single intersection, this time representing the Low state (dark red curve in Fig. 4c). Only at intermediate inhibitor amounts do two stable states emerge (red curve in Fig. 4c), illustrating the dependency of this bistable network on a specific parameter framework. Presuming fluctuation-free conditions, switching from the High state to the Low state when gradually increasing the inhibitor concentrations does not occur until the upper part of the expression curve is shifted (in direction of the x-coordinate) to not intersect the degradation line, and consequently only one single intersection remains: the Low state (Fig. 4c). Decreasing the inhibitor concentration again, however, triggers no instant switching back at the same inhibitor concentration. This is caused by different amounts of activator proteins present in the Low state. The effect of activator abundance, combined with the amount of inhibition, determines the promoter expression activity. The two critical inhibitor concentrations for triggering the switching consequently determine the input framework for the bistable system. This example also illustrates the history dependency of the described system, a phenomenon called hysteresis (Fig. 4d).
GENETIC ENGINEERING AND SYNTHETIC BIOLOGY

The ability to study complex gene regulatory networks, such as bistable switches, was rendered possible because of major advances in genetic engineering technology. For *S. pneumoniae*, genetic engineering is a “built-in by default” mechanism of the organism, facilitated by natural competence development. Historically, genetic engineering was discovered by Frederick Griffith in 192810, and it is ever since used to solve research questions. The continuous improvement of cloning techniques resulted in a paradigm shift of the application potential of genetic engineering, giving rise the field of synthetic biology. Synthetic biology aims at reprogramming cells towards novel functionalities and new applications100. To date, such reprogramming is only achieved for simple genetic circuits in model bacteria, because knowledge limitations still preclude from a complete understanding of how the genetic code, in combination with epigenetic factors, ultimately becomes translated into cell structure and cell behavior. Nevertheless, the first step towards making biology an engineering discipline was taken, and this thesis aims at contributing to this development by paving the way for synthetic biology applications in *S. pneumoniae*.

THESIS OUTLINE

Here, we report on synthetic biology studies with a focus on the important human pathogen *S. pneumoniae*. First, a cloning platform is presented, which allows for the standardized assembly of genetic elements (*Chapter 2*). Second, this system is used to build a selection platform, including selectable and counterselectable markers. Constitutive and inducible promoters are identified out of synthetic libraries, and gene expression circuits based on transcription repressors are constructed and characterized. These circuits include inverters, a logic AND gate and genetic toggle switches that give rise to bistable gene expression patterns (*Chapter 3*). An understanding of the dynamic impact of antibiotics was key for the construction of the selection platform, and preliminary experiments suggested that growth inhibition patterns correlate with the antibiotic classification (bacteriostatic or bactericidal). These observations sparked our interest in a more detailed study of antibiotic inhibition and resistance, including observations at the single-cell level (*Chapter 4*). We found that heterogeneity in antibiotic susceptibility and continued gene expression activity are important factors for resistance development. Furthermore, we found that the expression of chloramphenicol acetyltransferase in resistant cells protects susceptible cells that are present in the same environment. We show that this cross-protection establishes via intracellular antibiotic deactivation and demonstrate a potential clinical relevance of this mechanism (*Chapter 5*). Finally, we present a study of the dynamics of switching behavior of multistable systems, on the basis of the well-characterized lac-pathway of *E. coli* (*Chapter 6*).
References


