Engineering approaches to investigate pneumococcal gene expression regulation and antibiotic resistance development
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Chapter 7
Summary and Discussion

Summary of results
The research presented in this thesis aimed at paving the way for synthetic biology applications in *Streptococcus pneumoniae*. As a starting point, described in Chapter 2, the pneumococcal engineering platform PEP was created, which allows for BglBrick assembly of genetic components in form of translational units and expression units. The BglBrick cloning system was furthermore extended to enable the exchange of ribosome binding sites, the addition of N- an C-terminal peptide tags and the assembly of fusion proteins, giving rise to the BglFusion format. Three constitutive promoters (P1, P2 and P3) and the Zn\textsuperscript{2+}-inducible promoter PZ1 were assembled with the reporters *luc*, *gfp* and *mKate2*, and gene expression profiles were analyzed. These profiles were displayed, amongst others, in a double-logarithmic plot (normalized luminescence versus cell density) to show the growth phase dependency of gene expression activity. In Chapter 3, the cloning platform PEP was used to assemble a selection platform, consisting of a positive selection marker (*erm*), a negative selection marker (*pheS*), a population reporter (*luc*) and a single-cell reporter (*gfp*). This selection platform was used to screen combinatorial promoter libraries, resulting in the identification of constitutive promoters of predetermined strength. Furthermore, promoter regions were investigated for sequence variability. On the basis of these results, inducible promoters harboring operators for the repressors TetR and LacI were created and used to assemble gene regulatory networks including inverters, a logic AND gate and toggle switches.

In Chapter 4, studies of the antibiotic growth inhibition of the pneumococcus were presented, with a specific interest in the link between inhibition dynamics and the bactericidal activity of antimicrobial agents. We found that increasing concentrations of bacteriostatic antibiotics decreased the doubling-time of pneumococci immediately from the starting point of drug exposure; in contrast, for bactericidal antibiotics, increasing concentrations resulted in shortened periods of uninhibited growth before growth inhibition took effect. Furthermore, single-cell studies of antibiotic treatment were presented, and these studies elucidated, amongst others, that pneumococcal heteroresistance towards cell wall synthesis inhibitors develops upon induction, and in competition with ongoing cell lysis. Both cell-to-cell variability in antibiotic susceptibility and continuous gene expression activity during drug exposure were demonstrated to impact resistance.
development. In Chapter 6, a special characteristic of chloramphenicol resistance was investigated. We showed that the microbial context during infectious disease can compromise chloramphenicol therapy. In an environment containing CAT-expressing cells chloramphenicol becomes gradually deactivated via intracellular acetylation, resulting in the depletion of the antibiotic. Finally, in Chapter 7, the dynamics of switching events of bistable systems was assayed, on the basis of the lac-pathway in Escherichia coli. With the help of mathematical modelling, the rate-limiting fluctuations underlying switching events from the un-induced to the induced state were identified. Single-cell switching was shown to depend on the time period over which LacI repressor, after promoter dissociation, remains unbound from the lac promoter.

**Synthetic biology applications of the pneumococcus**

Synthetic biology aims at reprogramming biological systems, and at engineering organisms towards novel functionalities and new applications. In the context of the human pathogen S. pneumoniae, engineering attempts might pursue several goals. The proximate goal of pneumococcal engineering could represent support for fundamental research, such as to enable studies of gene expression patterns of higher complexity. The expression of virulence factors, for example, is regulated by multifactorial cues and can result in complex patterns including population bifurcations. The ability to reengineer such circuits would allow for in vivo studies of the importance of gene expression control on the pathogenicity of S. pneumoniae, an endeavor that was a major inspiration for this thesis (Chapters 2 and 3). The distal goal of pneumococcal engineering, to date merely a vision and a technological utopia, could be found in species-wide genome engineering attempts: the infiltration of S. pneumoniae by a genetic program that reduces pneumococcal pathogenicity. Engineering the genome of a human commensal (and opportunistic pathogen), also referred to as microbiome engineering\(^{12}\), raises many questions, of ethical nature, concerning human and environmental safety, and of technical feasibility. In the following, these issues shall be discussed in more detail, with a keen focus on the case of the pneumococcus.

The eradication of infectious disease is an ambitious goal that requires a global action plan, and these initiatives are spearheaded by the World Health Organization (WHO) which is an agency within the United Nations. The biggest success story to date is the Smallpox Eradication Program (1966-1980), resulting in the elimination of the disease in 1979. Current programs include the Global Polio Eradication Initiative (since 1988), while the Global Malaria Eradication Campaign was dropped in the 1970s because it was considered too ambitious. Requirements for the ability to eradicate a disease include the
absence of a non-human reservoir, the availability of effective clinical interventions, the ability to clearly identify carriers and the presence of a critical community size below which the pathogen ceases to circulate. In the case of the pneumococcus, only the first two criteria are fulfilled. However, eradicating the pneumococcus might be far more difficult than limiting or eliminating its pathogenicity. Microbiome engineering could allow for such measures, and the field of synthetic biology might develop and provide this technology in the near future.

In parallel to technical advances, a bioethics debate is required. The question ‘To what extent is it justifiable to actively intervene in the evolution of a species?’ was answered in the past with a strong bias depending on the species, as exemplified by protection initiatives, such as the International Union for the Conservation of Nature (IUCN) Red List of endangered species, in contrast to the mentioned eradication initiatives by the WHO. A more difficult to answer question is ‘How to weigh the benefits of microbiome engineering against potentially unpredictable negative consequences for human health and the environment, and which conflicts of interest must be taken into consideration?’. For the example of S. pneumoniae, with its high genomic plasticity, one could argue that anthropogenic microbiome manipulation already takes place for decades, by inadvertently selecting for antibiotic resistance and against vaccine serotypes (the rise of antibiotic resistance following widespread antibiotic usage and the changes of the composition of prevailing serotypes following large-scale PCV vaccination trials are discussed in Chapter 1). Nevertheless, microbiome engineering would represent a new dimension of human intervention, characterized by a more conscious and more goal-oriented manipulation, and furthermore by an execution on a shorter time scale.

The discussion of ethical issues of microbiome engineering will likely show similarities to the debate about genetically modified organisms (GMOs) in food, and the example discussed above (inadvertent versus conscious microbiome manipulation) resembles the appointed boundary between traditional breeding methods and GMO technologies. A distinguishing factor is that the spreading of GMO plants can be contained, within limits, and furthermore that products can be labeled and thus also chosen against by consumers. A genetically engineered microbiota, in contrast, would be more difficult to contain, resulting in an incapacitation of individuals whether to support this technology or not. However, the public opinion and support concerning GMOs might differ between red (medical) biotechnology applications and green (agricultural) biotechnology applications, depending on factors such as the verifiable and perceived benefit of the technology, transparency of the debate, education of the public, etc.
Benefits of microbiome engineering

The human microbiota, which is the aggregate of microorganisms residing on the skin, on the mucosa and in the gastrointestinal tract, is the result of eons of host-symbiont co-evolution. There is increasing awareness that the presence of an intact microbiota plays a crucial role for human health and wellbeing. The gut microbiota, for example, contributes to metabolic processes by breaking down nutrients that humans cannot digest. The commensal microbiota on epithelial surfaces contributes to the first line of defense against pathogens, both passively by imposing niche competition and actively via the production of antimicrobial peptides. Importantly, the presence of an intact microbiota guarantees regular communication between foreign cells and the immune system, which is believed to represent an essential contributing factor for the priming of immune responses\textsuperscript{4,5}. The dramatic changes of human ecology during the last century, including improved sanitary conditions and clinical interventions, likely caused disturbances to the evolved co-existence of humans and their microbiota\textsuperscript{6,7}.

People in modern society struggle from thitherto uncommon immune pathologies, such as allergic disease and autoimmune disorder including type 1 diabetes and multiple sclerosis\textsuperscript{8}. There is evidence that these phenomena are associated to alterations of the microbiota, caused by an impaired horizontal transfer, the hygiene hypothesis\textsuperscript{9}, and/or by an impaired vertical transfer, the disappearing microbiota hypothesis\textsuperscript{6}. For example, the progressive disappearance of the human commensal and opportunistic pathogen Helio\textit{bacter pylori} from its gastric niche was shown to be linked to increases of esophageal cancer and allergic asthma\textsuperscript{10}. The importance of symbionts for immune maturation could also be demonstrated on the basis of animal models; germ-free mice were shown to exhibit defective T and B cells in the mucosa, reduced T helper cells in peripheral lymphoid organs and diminished levels of IgG and IgA\textsuperscript{4}. Interestingly, all of these immune deficiencies could be reversed by microbial colonization\textsuperscript{4}.

Modern clinical interventions, in specific antibiotic therapy and vaccination, represented a revolution for human health. However, in the context of a more comprehensive health perspective, it might be necessary to reevaluate the unintended consequences of these interventions, such as the long-term effect of broad-spectrum antibiotic therapy on the microbiota or the alteration of the bacterial carriage after vaccination\textsuperscript{11}. To date, these clinical interventions are without alternative. Microbiome engineering, and in specific the engineering of an opportunistic pathogen towards reduced pathogenicity, might represent such an alternative, by solving a problem before it comes into existence. In this context, clinical interventions could become largely unnecessary and would only need to be applied in rare cases of infectious disease caused by non-
opportunistic pathogens. Reduced antibiotic usage, in turn, would not only allow for fewer disturbances of the microbiota, but furthermore contribute to the rehabilitation of effective antibiotic therapy (see Chapter 1).

Misusage of antibiotics during the last decades has resulted in the rise of antibiotic resistance, which is on the verge of becoming a serious threat for human health\textsuperscript{12}. This issue is especially problematic because of a lack of new antimicrobial agents on the market, and the search for new drugs remains an urgent task. However, even for drugs that were not discovered to date, resistance mechanisms already exist that will eventually spread. In all likelihood, it will be difficult to generate a sustainable system for antibiotic therapy, in which a specific antibiotic remains effective for a long period of time. Consequentially, new agents will need to be discovered and new therapies will need to be developed on a regular basis, in line with Lewis Carroll’s Red Queen’s race that ‘it takes all the running you can do to keep in the same place’. Microbiome engineering might represent a game changer to this race. Importantly, the technology could not only solve the issue of spreading resistance, but also overcome another major limitation of current clinical interventions: the limited access to antibiotic therapy and vaccinations of third-world country residents.

**How to evolve the pneumococcus**

Actively influencing the microbiota, although still controversial, has become an increasingly common therapeutic intervention, such as fecal microbiota transplants or the ‘seeding’ of newborns after C-section with vaginal swaps\textsuperscript{13–16}. However, in some cases, it might not be possible to replace the ‘bad bacteria’ by the ‘good bacteria’, because of evolutionary fitness constraints. Pathogens might thrive because of the very characteristic of being pathogenic, for example when sterile tissue can be accessed or when disease symptoms increase the host-to-host spreading (note that disease symptoms are often caused by the immune response, and not by the pathogen itself)\textsuperscript{17}. In the field of evolutionary biology, virulence was initially considered to represent the artifact of an evolutionary recent association between a host and a parasite\textsuperscript{18}. The reasoning behind this hypothesis was that harming the host, in the long run, would also harm the parasite depending on the host, and co-evolution should consequently drive the relationship towards commensalism. However, in more recent years, other hypothesis have been made which suggest that virulence, under certain conditions, can be maintained in the course of co-evolution\textsuperscript{18–21}.

The association between humans and \textit{S. pneumoniae} is believed to date back a long time, as evidenced by the appearance of \textit{iga} in response to the emerging immunoglobulin A1 (IgA1) subclass in the common lineage of humans, chimpanzees and gorillas\textsuperscript{22}. This
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raises the question why the pneumococcus, despite its long history of human association, did not lose its pathogenicity. Investigations of streptococcal evolution showed that the common ancestor of *Streptococcus pneumoniae* and its close relative *Streptococcus mitis* likely resembled the present-day pneumococcus\(^2^2\). Ancestral strains thus indeed have developed towards reduced virulence allowing for long-term carriage, giving rise to the non-pathogenic *S. mitis*. However, a second strategy seems to have proven successful, the ability to gain high genomic plasticity, resulting in *S. pneumoniae*\(^2^3\). The characteristic of high genomic plasticity enables the pneumococcus to reinfection individuals that have developed humoral immunity, provided that this immunity targets a different serotype. The fact that both strategies (decreased virulence and high genomic plasticity) developed in parallel indicates that different ecological niches have been occupied, disqualifying *S. mitis* as candidate for replacing *S. pneumoniae*.

Other contributing factors for the apparent present-day virulence of *S. pneumoniae* might be found in recent changes within the human host population, such as increased lifespans accompanied by a fading responsiveness of the immune system or decreased mortality of individuals with a weak immune systems because of clinical interventions. Regardless, pneumococcal virulence does not seem to represent a last remainder of an evolutionary adaptation process towards commensalism; the ability to induce and resist mucosal inflammations likely represents a fitness advantage that outweighs the negative consequence of a subsequent humoral immunity. This fitness advantage could be found, for example, in the clearance of niche competitors or in increased spreading via the stimulation of secretion production (Chapter 1). Causing systemic disease, however, does not contribute to pneumococcal spreading and rather represent a collateral damage that imposes a fitness burden (Chapter 1). Nevertheless, *S. pneumoniae* maintained its pathogenicity to date, indicating that the current lifestyle is a successful strategy that places the organism at a local peak within its evolutionary fitness landscape.

It seems unlikely that *S. pneumoniae*, on the time scale of decades, will be replaced from its ecological niche or that the organism will evolve towards reduced pathogenicity. Nevertheless, it appears reasonable to assume that the current pneumococcal colonization strategy that is accompanied by opportunistic pathogenicity can be improved, both from the perspective of humans (reduced morbidity and mortality of carriers) and from the perspective of *S. pneumoniae* (increased spreading via healthy carriers). Microbiome engineering could represent a way to fast-track such an evolution, and thus to bridge a valley in the fitness landscape of the pneumococcus, by infiltrating the organism with a synthetic genetic program. This program needs to fulfill three criteria: (i) reduce the
pathogenicity, the main goal; (ii) increase the fitness, to establish the program; (iii) intrinsically tie these two traits. The ability to cause mucosal inflammations of the nasopharynx should be maintained, while preventing from systemic infections, and evolved pneumococci carrying the genetic program need to be able to outcompete their pathogenic ancestors.

Preventing from systemic disease should represent a fitness advantage by itself, and one could, for example, construct a program on the basis of a toxin-antitoxin system, where the antitoxin is exclusively expressed in the nasopharynx. Such a network would show a similar functionality as tumor suppressor genes in mammalian cells, by preventing from harm caused by misguided individual cells to the overall cell population. Instead of responding to the location, one could also construct a system that monitors the responsiveness of the immune system, and that regulates virulence factor expression accordingly. Launching such programs likely imposes a manageable risk for human and environmental safety. However, the fitness increase accompanied by decreased human morbidity and mortality might not be sufficient for a species-wide establishment of such regulatory circuits. A direct advantage within the nasopharynx might be required, especially when considering the case of mixed infections (modified and non-modified pneumococci), to overcome the burden imposed by the expression and maintenance of the genetic program.

The fitness increase required for the spreading of a synthetic regulatory network could be enhanced in different ways, for example by antimicrobial peptide expression or via an external regulation on the basis of antibiotic resistance. However, it would be difficult to intrinsically tie such factors to a mechanism for reduced pathogenicity, and the two genetic traits might eventually uncouple. These programs consequently represent a high risk for human health and may give rise to unpredictable consequences. In any case, the discussed strategies will need to be examined with extreme care, by in vitro studies, animal infection models and mathematical modeling. Furthermore, transmission methods enabling horizontal gene transfer need to be inquired, a task that S. pneumoniae, with its high genomic plasticity and the ability to develop natural competence, might be well suited for. Together, the discussed preconditions for a reasonable implementation of microbiome engineering meet many of the characteristics of the pneumococcus, making the organism a potentially interesting candidate for exploring future real-life applications of synthetic biology.
References


