doi:10.2533/chimia.2020.402 Chimia 74 (2020) 402–406 © S. Panke

# Taming the Beast of Biology: Synthetic Biology and Biological Systems Engineering

Sven Panke*

*Abstract:* Despite the availability of a variety of '-omics' technologies to support the system-wide analysis of industrially relevant microorganisms, the manipulation of strains towards an economically relevant goal remains a challenge. Remarkably, our ability to catalogue the participants in and model ever more comprehensive aspects of a microorganism's physiology is now complemented by technologies that permanently expand the scope of engineering interventions that can be imagined. In fact, genome-wide editing and re-synthesis of microbial and even eukaryotic chromosomes have become widely applied methods. At the heart of this emerging system-wide engineering approach, often labelled 'Synthetic Biology', is the continuous improvement of large-scale DNA synthesis, which is put to two-fold use: (i) starting ever more ambitious efforts to re-write existing and coding novel molecular systems, and (ii) designing and constructing increasingly sophisticated library technologies, which has led to a renaissance of directed evolution in strain engineering. Here, we briefly review some of the critical concepts and technological stepping-stones of Synthetic Biology on its way to becoming a mature industrial technology.

**Keywords**: DNA synthesis · Orthogonal operating systems · Synthetic Biology · Systems assembly

***Sven Panke*** received his degree in Biotechnology from the Technical University of Braunschweig in 1995 after work at the German National Research Centre for Biotechnology (Braunschweig, with K. Timmis) and the Centro de Investigaciones Biologicas (Madrid, Spain, with V. de Lorenzo). He received his PhD in 1999 from ETH Zurich, Switzerland, for his work on the production of fine chemicals with recombinant bacteria with Bernard Witholt and Marcel Wubbolts. After a two-year stay in the biocatalysis group of the pharma product group of the Dutch chemical company DSM (Geleen, The Netherlands), he returned to ETH in 2001 as an Assistant Professor for Bioprocess Engineering. After receiving tenure in 2007, he moved to the newly founded ETH Department of Biosystems Science and Engineering in Basel. His main research topics include enzyme technology, high-throughput screening, and synthetic biology. His work was awarded with the ETH Medal and the DSM Research Award.

## 1. DNA Synthesis – The Machine Room of Synthetic Biology

Defining 'Synthetic Biology' is often a cumbersome effort, but one starting point to think about the topic is our increasing ability to do chemical (*i.e.* non-template driven) DNA synthesis. Although this is clearly a topic that appeared early on in the story of the progress of the life science enterprise,[1] it has been only much more recently that large-scale DNA synthesis has become a commodity that is within reach of many academic laboratories.[2]

In its essentials, the DNA synthesis methodology that underpins Synthetic Biology – phosphoramidite chemistry – has not changed since the mid-1980s. In a cycle of deprotecting the 5'-hydroxyl group at the end of a growing polynucleotide chain, growing the strand *via* the phosphoramidite linked to the 3'-hydroxyl-group of an incoming monomer, and oxidizing the phosphoramidite, DNA strands are grown into oligonucleotides of – roughly speaking – up to 50 nucleotides. In its standard implementation, this process is run on beads of controlled-pore glass, and the major sources of sequence errors in the recovered molecules are imperfect capping of growing strands that were not extended by coupling (leading to polynucleotides with deletions) and depurinations caused by residual acid remaining after the deprotection step. The resulting errors in the sequence of (some of) the obtained DNA molecules remain a weak point of the technology: although in absolute numbers the error rate is astonishingly low, it is too high to allow direct use of oligonucleotides in large-scale DNA synthesis projects in which several of these oligonucleotides need to be combined to a larger piece of DNA (see below). As a result, assembly products obtained from such oligonucleotides need to be sequence verified, at which point the process becomes inevitably slow and (relatively speaking) expensive.

Encouragingly, methods have been introduced that couple powerful next-generation sequencing technology to sequence verification of each single oligonucleotide and suggest that large-scale sequence verification in the future might be much more efficient.[3] More recently, also bead-based phosphoramidite chemistry has been increasingly complemented by methods that use similar chemistry in novel technological settings such as ink-jet printing.[4] These methods have a lower error rate, but deliver considerably less material than bead-based methods. Consequently, they are cheaper, and the amount of material is often sufficient to allow assembly,[5] or in any case to serve as a template for further amplification.

Once oligonucleotides are available, they can be easily assembled into larger fragments, often by a combination of thermal

*Correspondence:* Prof. S. Panke, E-mail: sven.panke@bsse.ethz.ch
Department of Biosystems Science and Engineering, ETH Zurich, Mattenstrasse 26, Zurich, Switzerland

cycling processes[6] and classical[7] or accelerated *in vitro* cloning methods.[8] When the target DNA molecule that needs to be assembled becomes too large, assembly is often transferred *in vivo*, *i.e.* into a suitable microorganism such as the yeast *Sacharomyces cerevisiae*, which is particularly gifted in assembling DNA molecules by homologous recombination,[9] or in which selection for successful assembly can be efficiently applied, such as the model bacterium *Escherichia coli*.[10] In fact, these methods have been successfully used to assemble genomes ranging from as small as 5.4 kbp for the phage ΦX174[11] *via* yeast chromosomes[12] to a 4 Mbp version of a chromosome of *E. coli*.[13]

While such chromosome synthesis projects illustrate the reach that template-free DNA synthesis has acquired, they only represent one aspect of the power of large-scale DNA synthesis. A second aspect is the design of libraries of unprecedented complexity and scope. For example, it is easily possible to acquire thousands of DNA oligonucleotides that have been synthesized, each according to its own pre-defined sequence specification *via* DNA printing.[4] This allows, for example, to overcome the notorious amino acid bias in directed evolution projects with proteins,[14] but also interrogating hundreds of specific sequence-based cellular features in strain engineering programs,[15] or the investigation of thousands of modularly composed peptides for antibiotic function.[16]

## 2. DNA Synthesis Meets Novel Experimental Methods

A part of the power of DNA synthesis stems from a variety of methods that have emerged over the last decades and that allow programming function in microorganisms to an extent that has not existed before. I will exemplify this with three methods that fit this description to an extraordinary degree: small RNA (sRNA)-based gene knockdown, multiplexed genome engineering, and CRISPR-Cas9-based genome engineering.

Large-scale sRNA engineering in the model bacterium *E. coli* was introduced in the laboratory of Sang Yup Lee[15] and revolves around the idea that one can design in a standardized fashion DNA genes for modular RNA molecules. These RNA molecules consist of two parts: one variable part that is complementary to an important part of an mRNA and one constant part that mediates interaction with the Hfq protein. The variable part has the potential to form double-stranded RNA structures with a specific mRNA, and in this state the function of the mRNA is (at least to some extent) blocked. The constant part recruits the RNA chaperone Hfq, which in turn stabilizes the double-stranded structures that are formed by the variable part. This way, specific messages can be sequestered from the pool of actively translated messages in a programmable fashion. By taking the variable parts from genes across the entire genome and expressing the corresponding sRNA genes alone or in combination in a suitable host, it becomes possible to engineer or investigate complex phenomena such as strain performance by concomitantly modulating a broad variety of cellular processes.[17]

Multiplexed genome engineering (MGE), again developed for *E. coli* but since then adapted for many different microorganisms, was introduced in the lab of George Church.[18] The idea is to introduce (freely programmable) oligonucleotides into a dividing cell. The cell can then use the oligonucleotide as an Okazaki-fragment in the DNA replication of the lagging strand during cell division, and this way a programmed mutation is introduced into a fraction of a population. Depending on the sequence of the oligonucleotide, everything from point mutations to large insertions and deletions can be introduced. Of course, the cell is equipped to fight this intervention, so a variety of measures to increase the efficiency of the process have to be taken,[19] but essentially MGE has become a standard method to systematically engineer bacterial genomes with unprecedented ease. Again, the oligonucleotide used for engineering can easily stem from a printed DNA-chip, and repeated transformations of the target strain with a library of mutagenic oligonucleotides can generate large numbers of strains with specific mutations, again enabling the modulation of quite complex phenotypes.[18] Even the problem of tracing the relevant mutation back to the critical oligonucleotide has been elegantly solved.[20]

Finally, the advent of the CRISPR/Cas9 technology has also transformed genome engineering in industrially relevant microorganisms. As a quick reminder, modular RNA molecules (or, depending on the implementation, two separate RNA molecules) interact with a DNA nuclease – often Cas9 – which derives its binding affinity for a specific DNA sequence from an RNA sequence that can be relatively freely (again, some constraints might apply[21]) programmed to direct the nuclease to a location of choice. The modular RNA molecules consist again of one constant part that directs the RNA molecule to the Cas9 nuclease and one variable part that determines at which sequence the nuclease binds and cleaves. If the nuclease is active, the method can be used to freely program a selection mechanism that selectively cleaves specific sequences from a pool. Combined with MGE, this becomes a very effective mechanism to enrich for cells that have undergone the desired mutation – by simply programming the Cas9 nuclease to bind to and then cleave DNA molecules that still have the unmodified DNA sequence.[22] If the nuclease is catalytically dead (but retains its binding properties), then the system can be used to build ‘road blocks’: repressor proteins that can be directed to many different places and thus – again – modulate complex phenotypes.[23]

In summary, the ability to write DNA is changing our ability to conduct experiments, in general, but also specifically in the domains of biotechnology and Synthetic Biology. There are two complementary main thrusts: Raw synthesis power simply allows coding ever more and more information, and library power improves our ability to ask increasingly sophisticated questions or to expand the scope of the question (*i.e.* to expand the number of genetic elements whose potential involvement in a specific process can be modulated in an experiment).

## 3. From Parts to Molecular Systems

Raw coding power is only helpful if it can be used meaningfully, and this challenge remains at the heart of Synthetic Biology research. Early strategies to facilitate the reliable programming of biosystems *via* synthetic DNA attempted to transfer methodological principles from classical engineering disciplines such as electrical engineering or computer science, including concepts such as composability and modularity. The idea was (and often still is) to find ways to integrate parts, maybe organized into ‘well-behaved’ modules, into the construction plan of a biosystem so that they behave more or less exactly as predicted based on their own properties. This would ensure function of the overall system by ‘composing’ it from functionally independent parts.

This approach has produced a full variety of promising engineering concepts, including adding self-cleaving non-translated ribozyme sequences 5' to single genes to make their expression less context depending[24] or the concept of the double open reading frame (ORF), in which a first (short) ORF is used to recruit a ribosome to an mRNA and thus eliminate all sorts of secondary structures, so that translation from an immediately following second ORF occurs exactly as predicted.[25] Such efforts were paired with increasingly comprehensive catalogues of ‘parts’ that allow increasingly precise fine tuning of cellular processes at various levels, including sets of promoter variants that differ in transcription initiation rates[26] and transcriptional terminators that differ in termination efficiency.[27] On the computational side, increasingly powerful programs in biological computer-aided design (‘BioCAD’) become available,[28] that make the overall effort from initial design to final DNA sequence much more manage-

able. Using these principles, quite remarkable design successes have been achieved, including the transfer of the ability to fix nitrogen from *Klebsiella oxytoca* to *E. coli* after testing around 500 constructed gene cluster variants.[29]

The importance of computational efforts can hardly be exaggerated in this context. Increasingly, BioCAD software integrates elements that support the assembly of DNA elements to encode a workable biosystem by integrating functional components – say, options that advise on how to construct a specific logic element based on a simulation built into the design program and going back to increasingly refined models for biological processes.[30] The increasing availability of useful models is a result of two developments that reinforce each other: on the one hand, the biological parameters that are required for using models for simulations (reaction rate constants, equilibrium constants, Hill coefficients …) become increasingly available as more and more biological processes are being investigated with unprecedented quantitative scrutiny. On the other hand, qualitative considerations regarding the structure of models increasingly allow making at least a qualitative statement about system behavior even if parameters remain scarce.[31]

Where reliable parameters are not available, physical models can help to obtain estimates. The most prominent example of a model with broad use in the bioengineering domain is probably the 'Salis ribosome binding site calculator' developed by Howard Salis in the laboratory of Chris Voigt.[32] Based on a physical model of the bacterial translation initiation process that was calibrated with a set of experimental data, the calculator allows associating a relative translation initiation rate with a specific DNA ribosome binding site (RBS) sequence and also to identify a DNA sequence for a desired relative translation initiation rate. Given the suitability of manipulating a short, defined DNA sequence such as the RBS with the current genome engineering technologies (see above), the calculator has received broad attention. However, despite its appeal and various efforts to refine it,[33] it remains an approximate tool rather than an exact predictor: whatever suggestion is provided needs to be confirmed experimentally. This is in fact the situation with many such design support models – the prediction is definitely useful, but not fully reliable. One way to deal with such scenarios is to combine computational prediction with DNA synthesis to generate small but smart libraries. Rather than one specific DNA sequence, the bioengineer generates a number of variants that is small enough to be experimentally accessible without major efforts but large enough to compensate for the insecurity of the prediction by testing different variants. The quality of the variant selection is ensured by using the physical model to remove non-functional variants from the very beginning.[34] Conceptually similar efforts to use small but smart libraries to deal with uncertainty developed around smart ways to improve strain performance by segregating long pathways into workable segments, optimizing those segments independently, and ideally supporting such efforts with design of experiments.[35]

A crucial component to such engineering efforts is of course standardization, without which large-scale projects cannot be undertaken. Here, Synthetic Biology has contributed to some quite remarkable developments. Early efforts still attempted to solve scaling problems in biosystems assembly. For example, the well-known iGEM 'idempotent' cloning standard[36] that strictly regulated the use of restriction enzymes in biosystems assembly was designed to enable recursive cloning, or in other words the assembly of large pieces of DNA by applying the same automation protocol over and over again. One particularly promising standard from the laboratory of Victor de Lorenzo is concerned with the consistent formatting of cloning vectors. The 'Standard European Vector Architecture' defines rules for plasmids with respect to restriction sites, overall plasmid architecture, and nomenclature that immensely facilitate the exchange of engineering tools.[37] Another very illustrative example is the regulation that was implemented to coordinate the Sc2.0 project, in the course of which all yeast chromosomes are to be replaced by fully synthetic chromosomes. All these chromosomes are expected to contain recombination sites for deletion of non-essential genes, instances of overlapping genes should be resolved, one stop codon should be lost, and tRNA genes should be relocated to one specific chromosome.[12]

Despite the different successes, it remains currently unclear how effective the effort to make biology 'engineerable' according to external engineering rules will turn out to be in the end. There are obvious issues such as evolution that might interfere with the longer-term stability of engineered biological systems. This issue has plagued strain development from the very beginning of modern biotechnology (say, the development of penicillin production strains), and the way to deal with such issues is to implement a robust system of master and working cell benches. Furthermore, efforts to streamline the *E. coli* genome have generated a strain that is actually more stable than standard strains can be expected to be, due to a complete absence of all sorts of mobile genetic elements.[38] Still, as designs become ever more complex and tend to build upon each other, it might become increasingly difficult to protect the integrity of all engineering modifications.

However, it remains also unclear how far we can actually reach in principle with the efforts described above towards rational biosystems design. As amazing as systems assembly efforts such as nitrogen fixation might be, already transferring the underlying system architecture (implemented in the form of the Cello programming language[28b]) from one Gram-negative model bacterium (*E. coli*) to another (*Pseudomonas putida*) puts an end to predictability [V. de Lorenzo, personal communication], suggesting poor robustness of our current efforts. Furthermore, the 'part' or 'pathway'-centric view that underpins many of the efforts discussed above consciously ignores that these systems operate in a context that defines boundary conditions and provides unexpected points of interference – the cell, or, in Synthetic Biology terminology, the 'chassis'.

## 4. The Chassis Problem

Of course there are reasons that the interactions between biomolecular system and chassis did not receive full attention from the very beginning, even though the tools of systems biology or systems biotechnology[39] might actually have helped also early on to understand certain limitations in design better. These reasons include that the potential of the 'part'-centric approach deserved to be fully explored, and that in fact it carried quite far. Still, from the point of view of 'predictability', it seems more and more pertinent to include holistic approaches into systems design. The tools and potential of systems biotechnology have been discussed many times, and therefore I will refer the reader to these publications to follow up in more detail.[40] Here, I will focus on complimentary strategies that are emerging in the Synthetic Biology community to increase predictability of engineered biomolecular systems.

One potentially very promising strategy includes the use of regulatory elements to limit the impact of an engineered biomolecular system (say, a pathway) on the physiology of the cell, or in other words, to ensure respecting specific performance limits dictated by the chassis. Such a strategy requires foremost a set of sensors that can record specific signals and then initiate certain regulatory processes to moderate the effects of the engineering design (say, reduce expression of pathways genes). What is a standard for many naturally available pathways is actually quite difficult to get right for synthetic pathways, as very little is available in terms of theoretical foundation or tools. However, recently quite a number of groups have started rectifying the dearth of sensor elements – typically by re-programming the specificity of existing transcriptional regulatory proteins – and of theoretical underpinnings. For

example, in a remarkable combination of theoretical and experimental work, the laboratory of Mustafa Khammash managed to construct an integral feedback bimolecular controller for which they theoretically proofed that their design was the only design possible and which they then implemented to control the specific growth rate in *E. coli*.[41] Also incoherent feed forward regulation was used to compensate for gene copy number variations in *E. coli* cells.[42] Next to this, also more classical strategies have been successfully implemented to limit pathway impact on the chassis, for example feedback inhibition that senses a critical metabolite in the cell and in response downregulates pathways leading to the metabolite's formation.[43] Clearly, such strategies cannot (yet) be simply programmed, their implementation still requires a lot of tinkering until the expression levels of the different participating molecular species are within a workable window of operation.

A completely different strategy emerges at the horizon of various current efforts in Synthetic Biology: the idea that one cell can contain two 'operating systems' that are actually separate from each other and therefore also no longer influence each other.[44] This idea revolves around the strategy of building increasingly complex 'parallel metabolisms' into a cell that interface with existing metabolism only at selected and controlled interfaces. Such interfaces arise at the metabolomic, the proteomic, or the genomic level. For example, different attempts are underway to introduce 'non-canonical' elements (such as fluorine) into metabolism[45] or to introduce novel cofactors to expand the array of available reaction mechanisms and hence the catalogue of cellular intermediates.[46] At protein level, new proteinogenic amino acids that expand protein function become available, for example to test biochemical hypotheses,[47] to facilitate chemical ligations *via* click chemistry, or allow tracking of proteins.[48] At the genomic level, two broad strategies are visible: on the one hand the attempt to make an *in vivo* alternative to DNA by complementing or replacing existing nucleotides by alternative molecules,[49] and on the other hand the effort to free up 'coding capacity' by liberating specific codons from coding duty. Such liberated codons that no longer code for a standard amino acid can then be occupied by reprogrammed tRNA/aminoacyl-tRNA synthetase systems that introduce non-canonical amino acids, facilitating the efforts to introduce novel proteinogenic amino acids. In fact, entire bacterial genomes have been liberated of one[50] or more[13] codons. The apparent ease and clarity with which nucleic acid-based interfaces can be re-programmed has in fact triggered many more efforts, for example in reprogramming the recognition elements between mRNAs and ribosomes, so that entire subpopulations of ribosomes can be 'reserved' for translation of specific messages.[51]

Despite the diversity of approaches, all these efforts align in testing the limits of extant biochemistry and, wherever possibility, to expand these, maybe even re-write them. It will be very interesting to see how far Synthetic Biology can develop this concept.

## 5. Synthetic Biology and the Litmus Test of Novel Technologies

In the spirit of this special edition of *CHIMIA* in memory of Oreste Ghisalba, it is easy to imagine what he might have asked if I had presented him with such a bouquet of possibly lofty ideas and concepts: "Is it useful?" Well, one way to answer this question is to look at adoption of the working principles of Synthetic Biology into industry. By that standard, Synthetic Biology is doing quite well. Some elements of its array of methods have found rapid assimilation into many companies that are concerned with strain construction in the broader sense, including the major chemical companies. But it is also encouraging to see that some of the more radical concepts have attracted large sums of money in the form of start-ups. While some early adopters such as Amyris (going back to the University of California at Berkeley and isoprene metabolism engineering in the laboratory of Jay Keasling[52]) and Synthetic Genomics (going back to the earlier genome synthesis efforts of efforts around Craig Venter[9]) had a bit of a mixed success, more recent endeavors such as Ginkgo Bioworks seem to be very successful in acquiring funding and attracting projects.[53] Complementing (and fueling) these efforts is a commercial DNA synthesis ecosystem in which competition and new technologies constantly reduce the cost of large scale DNA synthesis.[54] So in general, it is probably fair to say that Synthetic Biology has indeed been adopted into the world of commercial enterprise.

## 6. Outlook

In Switzerland, Synthetic Biology has grown very firm roots. As a major endeavor, ETH Zurich installed the Department for Biosystems Science and Engineering (D-BSSE) as a hotspot for Synthetic Biology in Basel. In the interdisciplinary environment provided by the department's different research pillars – experimental biology, computational biology, and engineering – Synthetic Biology thrives, with major advances in intracellular computation and cellular decision making (groups of Y. Benenson and M. Fussenegger[55]), genome editing (Platt group[56]), intracellular control strategies (Khammash group[41]), computationally supported biodesign (Stelling group[55a]), immunoengineering (Reddy-group[57]), and synthetic biochemistry (Panke group[58]). These efforts are complemented by many other prominent successes in Switzerland, for example in genome synthesis (Christen group[59]), genetic circuit engineering (Schaerli group[60]), cell-free engineering (Maerkl group[61]), biosensors (van der Meer group [62]), and transfer to industry, as exemplified by the company Evolva (*www.evolva.com*) and the spin-offs deepCDR Biologics (*www.deepcdr.com*), FGen (*www.fgen.ch*), and BioVersys (*www.bioversys.com*), which hopefully are only the first of a long list of future endeavors. So, yes, Switzerland is well prepared to take "the engineering of biology" to the next level and use it to address some of the grand challenges that we face in medicine and industrial and environmental sustainability. Very little of this would have been possible, had the field not been so expertly tilled before by many of my co-authors in this special issue and, above all, by Oreste Ghisalba, who tirelessly and with a clear view of the future worked towards making biotechnology prosper in Switzerland. As one of my colleagues, Philippe Marlière, keeps reminding me – and rarely has this been truer than here – we are all standing on the shoulders of giants. Thank you, Oreste.

***Acknowledgements***

The author gratefully acknowledges Synthetic Biology-related funding from the ETH Zurich, the Swiss National Science Foundation, the European Union, and Innosuisse.

Received: March 12, 2020

[1] K. L. Agarwal, H. Buchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, U. L. Rajbhandary, J. H. Van de Sande, V. Sgaramella, H. Weber, T. Yamada, *Nature* **1970**, *227*, 27, DOI: 10.1038/227027a0.

[2] M. H. Caruthers, *Biochem. Soc. Trans.* **2011**, *39*, 575, DOI: 10.1042/BST0390575.

[3] M. Matzas, P. F. Stähler, N. Kefer, N. Siebelt, V. Boisguérin, J. T. Leonard, A. Keller, P. H. Cord F. Stähler, B. Gharizadeh, F. Babrzadeh, G. M. Church, *Nat. Biotechnol.* **2010**, *28*, 1291.

[4] E. M. LeProust, B. J. Peck, K. S. H. B. McCuen, B. Moore, E. Namsaraev, M. H. Caruthers, *Nucl. Acids Res.* **2010**, *38*, 2522.

[5] S. Kosuri, N. Eroshenko, E. M. LeProust, M. Super, J. Way, J. B. Li, G. M. Church, *Nat. Biotechnol.* **2010**, *28*, 1295, DOI: 10.1038/nbt.1716.

[6] W. P. Stemmer, A. Crameri, K. D. Ha, T. M. Brennan, H. L. Heyneker, *Gene* **1995**, *164*, 49.

[7] S. J. Kodumal, K. G. Patel, R. Reid, H. G. Menzella, M. Welch, D. V. Santi, *Proc. Natl Acad. Sci USA* **2004**, *101*, 15573.

[8] D. G. Gibson, G. Benders, C. Andrews-Pfannkoch, E. A. Denisova, H. Baden-Tillson, J. Zaveri, T. B. Stockwell, A. Brownley, D. W. Thomas, M. A. Algire, C. Merryman, L. Young, V. N. Noskov, J. I. Glass, J. C. Venter, C. A. Hutchison III, H. O. Smith, *Science* **2008**, *319*, 1215, DOI: 10.1126/science.1151721.
[9] D. G. Gibson, J. I. Glass, C. Lartigue, V. N. Noskov, R.-Y. Chuang, M. A. Algire, G. A. Benders, M. G. Montague, L. Ma, M. M. Moodie, C. Merryman, S. Vashee, R. Krishnakumar, N. Assad-Garcia, C. Andrews-Pfannkoch, E. A. Denisova, L. Young, Z.-Q. Qi, T. H. Segall-Shapiro, C. H. Calvey, P. P. Parmar, C. A. Hutchison, H. O. Smith, J. C. Venter, *Science* **2010**, *329*, 52, DOI: 10.1126/science.1190719.
[10] K. Wang, J. Fredens, S. F. Brunner, S. H. Kim, T. Chia, J. W. Chin, *Nature* **2016**, *539*, 59, DOI: 10.1038/nature20124.
[11] H. O. Smith, C. A. Hutchison, 3rd, C. Pfannkoch, J. C. Venter, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15440.
[12] S. M. Richardson, L. A. Mitchell, G. Stracquadanio, K. Yang, J. S. Dymond, J. E. DiCarlo, D. Lee, C. L. V. Huang, S. Chandrasegaran, Y. Cai, J. D. Boeke, J. S. Bader, *Science* **2017**, *355*, 1040, DOI: 10.1126/science.aaf4557.
[13] J. Fredens, K. Wang, D. d. l. Torre, L. F. H. Funke, W. E. Robertson, Y. Christova, T. Chia, W. H. Schmied, D. L. Dunkelmann, V. Beránek, C. Uttamapinant, A. G. Llamazares, T. S. Elliott, J. W. Chin, *Nature* **2019**, *569*, 514, DOI: 10.1038/s41586-019-1192-5.
[14] A. Li, Z. Sun, M. T. Reetz, *ChemBioChem* **2018**, *19*, 2023, DOI: 10.1002/cbic.201800339.
[15] D. Na, S. M. Yoo, H. Chung, H. Park, J. H. Park, S. Y. Lee, *Nat. Biotechnol.* **2013**, *31*, 170, DOI: 10.1038/nbt.2461.
[16] S. Schmitt, M. Montalban-Lopez, D. Peterhoff, J. Deng, R. Wagner, M. Held, O. P. Kuipers, S. Panke, *Nat. Chem. Biol.* **2019**, *15*, 437, DOI: 10.1038/s41589-019-0250-5.
[17] a) D. Yang, S. M. Yoo, C. Gu, J. Y. Ryu, J. E. Lee, S. Y. Lee, *Metabol. Engin.* **2019**, *54*, 180, DOI: 10.1016/j.ymben.2019.04.003; b) M. Noh, S. M. Yoo, W. J. Kim, S. Y. Lee, *Cell Sys.* **2017**, *5*, 418, DOI: 10.1016/j.cels.2017.08.016.
[18] H. H. Wang, F. J. Isaacs, P. A. Carr, Z. Z. Sun, G. Xu, C. R. Forest, G. M. Church, *Nature* **2009**, *460*, 894, DOI: 10.1038/nature08187.
[19] A. Nyerges, B. Csoergo, I. Nagy, B. Balint, P. Bihari, V. Lazar, G. Apjok, K. Umenhoffer, B. Bogos, G. Posfai, C. Pal, *Proc. Natl Acad. Sci. USA* **2016**, *113*, 2502, DOI: 10.1073/pnas.1520040113.
[20] A. D. Garst, M. C. Bassalo, G. Pines, S. A. Lynch, A. L. Halweg-Edwards, R. Liu, L. Liang, Z. Wang, R. Zeitoun, W. G. Alexander, R. T. Gill, *Nat. Biotechnol.* **2017**, *35*, 48, DOI: 10.1038/nbt.3718.
[21] A. A. Dominguez, W. A. Lim, L. S. Qi, *Nat. Rev. Mol. Cell Biol.* **2016**, *17*, 5, DOI: 10.1038/nrm.2015.2.
[22] W. Jiang, D. Bikard, D. Cox, F. Zhang, L. A. Marraffini, *Nat. Biotechnol.* **2013**, *31*, 233, DOI: 10.1038/nbt.2508.
[23] L. S. Qi, M. H. Larson, L. A. Gilbert, J. A. Doudna, J. S. Weissman, A. P. Arkin, W. A. Lim, *Cell* **2013**, *152*, 1173, DOI: 10.1016/j.cell.2013.02.022.
[24] C. Lou, B. Stanton, Y.-J. Chen, B. Munsky, C. A. Voigt, *Nat. Biotechnol.* **2012**, *30*, 1137, DOI: 10.1038/nbt.2401.
[25] V. K. Mutalik, J. C. Guimaraes, G. Cambray, C. Lam, M. J. Christoffersen, Q.-A. Mai, A. B. Tran, M. Paull, J. D. Keasling, A. P. Arkin, D. Endy, *Nat. Meth.* **2013**, *10*, 354, DOI: 10.1038/NMETH.2404.
[26] a) H. Alper, C. Fischer, E. Nevoigt, G. Stephanopoulos, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 12678, DOI: 10.1073/pnas.0504604102 ; b) J. H. Davis, A. J. Rubin, R. T. Sauer, *Nucl. Acids Res.* **2011**, *39*, 1131.
[27] Y.-J. Chen, P. Liu, A. A. K. Nielsen, J. A. N. Brophy, K. Clancy, T. Peterson, C. A. Voigt, *Nat. Meth.* **2013**, *10*, 659, DOI: 10.1038/NMETH.2515.
[28] a) J. Beal, R. Weiss, D. Densmore, A. Adler, E. Appleton, J. Babb, S. Bhatia, N. Davidsohn, T. Haddock, J. Loyall, R. Schantz, V. Vasilev, F. Yaman, *ACS Syn. Bio.* **2012**, *1*, 317, DOI: 10.1021/sb300030d; b) A. A. K. Nielsen, B. S. Der, J. Shin, P. Vaidyanathan, V. Paralanov, E. A. Strychalski, D. Ross, D. Densmore, C. A. Voigt, *Science* **2016**, *352*, 53, DOI: 10.1126/science.aac7341.
[29] M. J. Smanski, S. Bhatia, D. Zhao, Y. Park, L. B. A. Woodruff, G. Giannoukos, D. Ciulla, M. Busby, J. Calderon, R. Nicol, D. B. Gordon, D. Densmore, C. A. Voigt, *Nat. Biotechnol.* **2014**, *32*, 1241, DOI: 10.1038/nbt.3063.
[30] B. Yordanov, N. Dalchau, P. K. Grant, M. Pedersen, S. Emmott, J. Haseloff, A. Phillips, *ACS Syn. Bio.* **2014**, *3*, 578, DOI: 10.1021/sb400152n.
[31] a) E. Karamasioti, C. Lormeau, J. Stelling, *Mol. Sys. Des. Engin.* **2017**, *2*, 410, DOI: 10.1039/c7me00032d; b) L. A. Widmer, J. Stelling, *Curr. Opin. Biotechnol.* **2018**, *52*, 17, DOI: 10.1016/j.copbio.2018.02.005.
[32] H. M. Salis, E. A. Mirsky, C. A. Voigt, *Nat. Biotechnol.* **2009**, *27*, 946, DOI: 10.1038/nbt.1568.
[33] A. E. Borujeni, A. S. Channarasappa, H. M. Salis, *Nucl. Acids Res.* **2014**, *42*, 2646, DOI: 10.1093/nar/gkt1139.
[34] M. Jeschek, D. Gerngross, S. Panke, *Nat. Commun.* **2016**, *7*, 11163, DOI: 10.1038/ncomms11163.
[35] P. K. Ajikumar, W.-H. Xiao, K. E. J. Tyo, Y. Wang, F. Simeon, E. Leonard, O. Mucha, T. H. Phon, B. Pfeifer, G. Stephanopoulos, *Science* **2010**, *330*, 70, DOI: 10.1126/science.1191652.
[36] B. Canton, A. Labno, D. Endy, *Nat. Biotechnol.* **2008**, *26*, 787, DOI: 10.1038/nbt1413.
[37] E. Martinez-Garcia, T. Aparicio, A. Goni-Moreno, S. Fraile, V. de Lorenzo, *Nucl. Acids Res.* **2015**, *43*, D1183, DOI: 10.1093/nar/gku1114.
[38] K. Umenhoffer, G. Draskovits, A. Nyerges, I. Karcagi, B. Bogos, E. Timar, B. Csoergo, R. Herczeg, I. Nagy, T. Feher, C. Pal, G. Posfai, *ACS Syn. Bio.* **2017**, *6*, 1471, DOI: 10.1021/acssynbio.6b00378.
[39] S. Y. Lee, D. Y. Lee, T. Y. Kim, *Trends in Biotechnol.* **2005**, *23*, 349, DOI: 10.1016/j.tibtech.2005.05.003.
[40] K. R. Choi, W. D. Jang, D. Yang, J. S. Cho, D. Park, S. Y. Lee, *Trends in Biotechnol.* **2019**, *37*, 817, DOI: 10.1016/j.tibtech.2019.01.003.
[41] S. K. Aoki, G. Lillacci, A. Gupta, A. Baumschlager, D. Schweingruber, M. Khammash, *Nature* **2019**, *570*, 533, DOI: 10.1038/s41586-019-1321-1.
[42] T. H. Segall-Shapiro, E. D. Sontag, C. A. Voigt, *Nat. Biotechnol.* **2018**, *36*, 352, DOI: 10.1038/nbt.4111.
[43] a) F. Zhang, J. M. Carothers, J. D. Keasling, *Nat. Biotechnol.* **2012**, *30*, 354, DOI: 10.1038/nbt.2149; b) R. H. Dahl, F. Zhang, J. Alonso-Gutierrez, E. Baidoo, T. S. Batth, A. M. Redding-Johanson, C. J. Petzold, A. Mukhopadhyay, T. S. Lee, P. D. Adams, J. D. Keasling, *Nat. Biotechnol.* **2013**, *31*, 1039, DOI: 10.1038/nbt.2689.
[44] C. C. Liu, M. C. Jewett, J. W. Chin, C. A. Voigt, *Nat. Chem. Bio.* **2018**, *14*, 103, DOI: 10.1038/nchembio.2554.
[45] L. Martinelli, P. I. Nikel, *Microb. Biotechnol.* **2019**, *12*, 187, DOI: 10.1111/1751-7915.13372.
[46] M. Jeschek, S. Panke, T. R. Ward, *Trends in Biotechnol.* **2018**, *36*, 60, DOI: 10.1016/j.tibtech.2017.10.003.
[47] N. Huguenin-Dezot, D. A. Alonzo, G. W. Heberlig, M. Mahesh, D. P. Nguyen, M. H. Dornan, C. N. Boddy, T. M. Schmeing, J. W. Chin, *Nature* **2019**, *565*, DOI: 10.1038/s41586-018-0781-z.
[48] L. Wang, J. Xie, P. G. Schultz, *Annu. Rev. Biophys. Biomol. Struct.* **2006**, *35*, 225, DOI: 10.1146/annurev.biophys.35.101105.121507
[49] a) D. A. Malyshev, K. Dhami, T. Lavergne, T. Chen, N. Dai, J. M. Foster, I. R. Correa, Jr., F. E. Romesberg, *Nature* **2014**, *509*, 385, DOI: 10.1038/nature13314; b) P. Marliere, J. Patrouix, V. Döring, P. Herdewijn, S. Tricot, M. Bouzon, R. Mutzel, *Angew. Chem. Int. Ed.* **2011**, *50*, 7109 DOI: 10.1002/anie.201100535.
[50] F. J. Isaacs, P. A. Carr, H. H. Wang, M. J. Lajoie, B. Sterling, L. Kraal, A. C. Tolonen, T. A. Gianoulis, D. B. Goodman, N. B. Reppas, C. J. Emig, D. Bang, S. J. Hwang, M. C. Jewett, J. M. Jacobson, G. M. Church, *Science* **2011**, *333*, 348.
[51] a) K. Wang, H. Neumann, S. Y. Peak-Chew, J. W. Chin, *Nat. Biotechnol.* **2007**, *25*, 770, DOI: 10.1038/nbt1314; b) H. Neumann, K. Wang, L. Davis, M. Garcia-Alai, J. W. Chin, *Nature* **2010**, *464*, 441.
[52] D. K. Ro, E. M. Paradise, M. Ouellet, K. J. Fisher, K. L. Newman, J. M. Ndungu, K. A. Ho, R. A. Eachus, T. S. Ham, J. Kirby, M. C. Y. Chang, S. T. Withers, Y. Shiba, R. Sarpong, J. D. Keasling, *Nature* **2006**, *440*, 940.
[53] J. Kelly, *ACS Syn. Bio.* **2012**, *1*, 4, DOI: 10.1021/sb2000287.
[54] A. L. Halweg-Edwards, W. C. Grau, J. D. Winkler, A. D. Garst, R. T. Gill, *Curr. Opin. Chem. Bio.* **2015**, *28*, 150, DOI: 10.1016/j.cbpa.2015.07.009.
[55] a) M. Xie, H. Ye, H. Wang, G. C.-E. Hamri, C. Lormeau, P. Saxena, J. Stelling, M. Fussenegger, *Science* **2016**, *354*, 1296, DOI: 10.1126/science.aaf4006; b) Z. Xie, L. Wroblewska, L. Prochazka, R. Weiss, Y. Benenson, *Science* **2011**, *333*, 1307, DOI: 10.1126/science.1205527.
[56] F. Schmidt, M. Y. Cherepkova, R. J. Platt, *Nature* **2018**, *562*, 380, DOI: 10.1038/s41586-018-0569-1.
[57] E. Miho, R. Roskar, V. Greiff, S. T. Reddy, *Nat. Commun.* **2019**, *10*, 1321, DOI: 10.1038/s41467-019-09278-8.
[58] M. Jeschek, R. Reuter, T. Heinisch, C. Trindler, J. Klehr, S. Panke, T. R. Ward, *Nature* **2016**, *537*, 661, DOI: 10.1038/nature19114.
[59] J. E. Venetz, L. Del Medico, A. Wolfle, P. Schachle, Y. Bucher, D. Appert, F. Tschan, C. E. Flores-Tinoco, M. van Kooten, R. Guennoun, S. Deutsch, M. Christen, B. Christen, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 8070, DOI: 10.1073/pnas.1818259116.
[60] Y. Schaerli, A. Jimenez, J. M. Duarte, L. Mihajlovic, J. Renggli, M. Isalan, J. Sharpe, A. Wagner, *Mol. Sys. Bio.* **2018**, *14*, e8102, DOI: 10.15252/msb.20178102.
[61] X. Wan, F. Volpetti, E. Petrova, C. French, S. J. Maerkl, B. Wang, *Nat. Chem. Bio.* **2019**, *15*, 540, DOI: 10.1038/s41589-019-0244-3.
[62] J. R. van der Meer, S. Belkin, *Nat. Rev. Microbio.* **2010**, *8*, 511, DOI: 10.1038/nrmicro2392.

### *License and Terms*

This is an Open Access article under the terms of the Creative Commons Attribution License CC BY_NC 4.0. The material may not be used for commercial purposes.

The license is subject to the CHIMIA terms and conditions: (*http://chimia.ch/component/sppagebuilder/?view=page&id=12*).

The definitive version of this article is the electronic one that can be found at doi:10.2533/chimia.2020.402