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Crystal ball – 2011

In this feature, leading researchers in the field of microbial biotechnology speculate on the technical and conceptual developments that will drive innovative research and open new vistas over the next few years.

Bioproducts from undefined mixed cultures: electron pushing

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A thermodynamic state analysis can inform the bioprocess engineer if a biological redox reaction is energetically feasible. This understanding is especially pertinent in complex systems, such as undefined mixed cultures that convert organic wastes, as a result of the many possible pathways that could operate in sequence or in parallel (Angenent *et al.*, 2004; Kleerebezem and van Loosdrecht, 2007; Agler *et al.*, 2011). In theory, the bioprocessing of organic substrates in an open microbial community-based process will result in the production of the end-product with the lowest free energy content per electron. This state can be considered as the thermodynamic equilibrium state and guarantees a maximum amount of free energy to be harvested by the microbial community catalysing the process (Hanselmann, 1991). In the presence of a strong electron acceptor, such as oxygen, the end-product of organic carbon conversion is carbon dioxide (0 kJ e-mol^{-1}, reference state) via complete mineralization. In the absence of an electron acceptor or external energy source, the end-product is methane (–23.0 kJ e-mol^{-1}). Combined with the *in situ* product separation of a gaseous end-product, these thermodynamic properties provide a firm basis for the anaerobic digestion process. Full-scale anaerobic digesters are, therefore, widely used to treat a wide range of solid substrates and wastewaters and the generated biogas is used for heat and electric power production – of note are the ~30 million domestic digesters in China. Still, despite these intrinsic advantages, methane is not the most valuable end-product because as a gas it has a lower energy density and is harder to store/transport than a liquid fuel. For this reason, bioprocess engineers are now looking for ways to produce more valuable liquid fuels or chemical building blocks from organic residues.

To produce a product that is more valuable than methane under anaerobic conditions with undefined mixed cultures, methanogenesis should be prevented. If aceticlastic methanogens are inhibited by, for example, lowering the pH from ~7 to ~5.8, acetate (–26.9 kJ e-mol^{-1}) accumulates because hydrogenotrophic methanogens still maintain low hydrogen partial pressures, allowing for anaerobic oxidation of intermediately formed short-chain carboxylates and ethanol. A further decrease in pH will inhibit hydrogenotrophic methanogens as well, and will shift the fermentation end-product spectrum to a mixture of carboxylates (e.g. propionate: –27.0 kJ e-mol^{-1}; and butyrate: –27.1 kJ e-mol^{-1}) and ethanol (–30.5 kJ e-mol^{-1}). At longer cell residence times, n-butyrate is often found at high relative ratios within the fermentation product mixture. At shorter residence times and with ready degradable substrates, lactate (–31.6 kJ e-mol^{-1}) dominates in some cases.

The environmental conditions in the reactor are such that these fermentation end-products, such as n-butyrate and lactate, cannot be further oxidized anaerobically. The production of short-chain carboxylates at relatively low concentrations (< 50 g l^{-1}), however, is not very attractive because it requires major efforts to recover them from the fermentation broth. The future quest in development of bioprocesses for fuel or building blocks should, therefore, aim at products that require a limited effort for product recovery. To date, *in situ* product separation has been accomplished by generating intracellular storage polymers (polyhydroxyalkanoates) after aerobic conversion of carboxylates, but new anaerobic production pathways are currently under investigation as well. These novel production routes reduce short-chain carboxylates to alcohols or medium/long chain fatty acids that can be separated from the fermentation broth by precipitation, distillation, or by concentration in an organic solvent. We believe that the reduction of carboxylates (also called biohydrogenation or chain elongation) by pushing electrons into the undefined mixed culture offers a prosperous route.

In situ upgrading of fermentation end-products by pushing with external reducing power (electrons) is envisioned to produce:

(i) a single main end-product rather than a mixture; and
(ii) a compound that can be separated from the fermentation broth.

Recently, a ground-breaking study by Steinbusch and colleagues (2010) demonstrated the production of

n-caproate [i.e. hexanoate (–27.2 kJ e-mol^{-1})] with an undefined mixed culture under anaerobic conditions after complete inhibition of methanogenesis with an antibiotic compound. The microbial communities catalysed a biological two-carbon chain-elongation reaction with n-butyrate to form n-caproate, using electrons from externally supplied ethanol that was oxidized to acetate. Importantly, this C6 carboxylate is relatively easy to separate from water because of its maximum solubility of ~10 g l^{-1} at 30°C. To generate a valuable fuel, the extracted n-caproate with an energy density of –144.8 kJ C^{-1}, should be further upgraded to, for example, n-hexanol with an energy density similar to ethanol and n-butanol (–182.7 and –171.4 kJ C^{-1}); or even better alkanes (–178.6 kJ C^{-1}), by additional external reactions.

Future research should focus on different methods to accomplish electron pushing: electrons can be supplied in many forms other than ethanol, such as by adding synthesis gas (syngas: mainly hydrogen and carbon monoxide); or directly at the cathode in bioelectrochemical systems. Particularly interesting of the latter system is that energy levels at which electrons are introduced can be manipulated by the power supply, or that hydrogen gas can be generated within the bioprocess at the cathode. Recently, researchers have demonstrated that organic molecules can be produced from carbon dioxide in a biocathodic compartment, whereas the electrons were generated from organic waste oxidation in the bioanodic compartment (Clauwaert *et al*., 2008).

Thermodynamic estimates can help to predict which chemicals may accumulate when an external electron donor is supplied. Molecular hydrogen is a stronger electron donor (–40 kJ e-mol^{-1}) than most (but not all) organic compounds, and therefore the net driving force in the system still aims for production of organic carbon in its most reduced form (methane). Upon selective inhibition of methanogenesis, as suggested above, there is a driving force for the production of other strongly reduced organic molecules. We also want to point out that, most likely, the production of strongly reduced compounds will be stimulated by carbon limited operational conditions. In addition, the end-product composition will depend strongly on the microbial community structure in the system and the extent to which the microbes are capable of harvesting the small amounts of energy available for the electron pushing reactions. Classical tools can be used to drive the process in a required product direction, such as bioaugmentation, adaptive evolution or selecting specific process conditions. Finally, ecology theory should be applied to synthesize communities that are stable to perturbations. After accomplishing these tasks successfully, we predict that undefined mixed cultures (carboxylate platform) will be integrated within a biorefinery concept with other platforms, such as sugar platform (ethanol), syngas platform (hydrogen and carbon monoxide) or renewable electricity platform (electrons) to generate bioproducts that can be upgraded further to bulk chemicals or liquid fuels.

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Looking through the crystal ball: where will our next generation drugs come from?

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Starting from recombinant DNA and production of industrial quantities of human insulin by gene cloning in *Escherichia coli* to the present day synthetic biology through chemical synthesis of a complete genome and introducing it in specific *Mycoplasma* strains or the role of gut microflora in obesity and other modern day illnesses, microbial biotechnology has come a long way. A classical example of the role of soil microorganisms in alleviating diseases is the production of antibiotics giving rise to a huge anti-infective industry. With the decline in the search for new antibiotics, and with the emergence of new infectious agents, or even aggressive forms of cancer, it is imperative that we seek help from bacteria, particularly the difficult to treat pathogenic bacteria causing chronic infections and having long-term residence in the human body, for fighting the life-threatening diseases.

Take for example the case of cancers, HIV/AIDS, malaria or tuberculosis, for which, in spite of years of research, no good drugs or vaccines exist. This is because these disease agents are very smart and quickly change the target(s) for the drugs or the epitope(s) for vaccines. The current technology used by the pharmaceutical industry is to rationally design inhibitors as drugs

against various single targets that are important for disease progression. Similar is the case for targeting key surface epitopes in pathogens for eliciting neutralizing antibodies as candidate vaccines. The pathogens quickly change such targets or epitopes, thereby becoming drug-resistant or making the vaccine ineffective. The pharmaceutical industries' solution is to use cocktails or combination of drugs, as in the treatment of HIV/AIDS or cancers, leading to high costs of treatment and potential problems of multi-drug resistance.

So what's the solution? What's needed are not rationally designed single-target drugs or vaccines directed towards key components of the pathogen, but intelligently designed drugs that will target multiple components of the disease agents, as well as block the host functions that are required for pathogen entry and propagation. That's not easy to design and is beyond our pharmaceutical industry's reach. That's where the evolutionary wisdoms of bacteria come in.

It is now becoming apparent that bacteria, particularly certain pathogenic bacteria that cause chronic infections in human bodies by forming biofilms on epithelial cell surfaces and establishing some kind of symbiotic relationship and long-term residence, possess the evolutionary wisdom of 3 billion years. Such bacteria, *Pseudomonas aeruginosa* for example, during chronic infections consider the host body as their habitat and try to protect their turf from other invading disease agents that can cause harm or kill the host, thereby depriving the bacteria of their sanctuary. Such invaders could be cancers, viruses such as HIV/AIDS or parasites such as the malarial parasite *Plasmodium falciparum* or the toxoplasmosis-causing parasite *Toxoplasma gondii*. To keep such invaders in check, *P. aeruginosa* has developed an exquisitely and intelligently designed weapon, a copper-containing periplasmic protein termed azurin, which is released when *P. aeruginosa* is exposed to its enemy such as a cancer. On release, the weapon azurin enters preferentially to cancer cells and stops them on their tracks by interfering in multiple steps including inhibition of cellular signalling, inhibition of angiogenesis and induction of apoptosis (Fialho and Chakrabarty, 2010). The same weapon is also released when *P. aeruginosa* is exposed to AIDS-causing HIV-1 virus, and significantly inhibits viral growth by not only binding the viral envelope protein gp120, but also host proteins CD4, ICAM-3 and DC-SIGN (Fialho and Chakrabarty, 2010). If the host receptors or transport agents are blocked, and if the interference is at multiple steps, the disease agents such as cancers or the HIV/AIDS virus will have problems eliciting resistance to such a bacterial weapon. Unlike *Meningococci* or *Gonococci* that cause infections in brain meninges, *P. aeruginosa* is not known to reach the brain. It is thus interesting to note that the *Meningococci* or *Gonococci* have deliberately modified their azurin-like weapon, called Laz, which has an additional 39 amino acid moiety in the N-terminal of azurin, called an H.8 epitope. This epitope allows Laz to cross the blood–brain barrier to fight brain tumours such as glioblastomas (Hong *et al*., 2010), and potentially allowing other drugs to cross the blood–brain barrier for the treatment of brain-related pathologies (Hong *et al*., 2010). Thus the weapons are intelligently designed and appropriately modified to attack the invaders depending on the bacteria's habitat.

Two questions!!! Because there are many cancers, many viruses and many parasites, is azurin the only weapon that *P. aeruginosa* uses to keep various invaders in check, or does *P. aeruginosa* use other weapons as well? Also, is *P. aeruginosa* the only bacterium that produces protein weapons to keep multiple disease agents in check or are there other bacteria that produce similar weapons? We have shown that *P. aeruginosa* produces other proteins with anticancer activity, such as arginine deiminase or Pa-CARD (Fialho and Chakrabarty, 2010), and we, as well as a group in Portugal, have isolated another secreted protein from another pathogenic bacterium that demonstrates anticancer and anti-HIV/AIDS activity, clearly suggesting that bacterial protein weapons, or peptides derived from them, could potentially be our next generation promiscuous drugs, where a single drug targets such major diseases as cancers, HIV/AIDS and others.

It is also interesting to point out that a single candidate drug such as azurin not only has activity against cancers, HIV-1 virus, *P. falciparum* and *T. gondii*, thereby potentially being useful in the treatment of AIDS patients with sarcomas or co-infected with malarial and other parasites, but azurin can also prevent the emergence of cancer (Das Gupta and Chakrabarty, 2009). This is very important in preventing the relapse of a cancer, after the cancer is treated, requiring the patient to take azurin lifelong as a preventive measure. Eleven US patents and many international patents have been issued to the University of Illinois to cover the potential utility of Laz, azurin, and an azurin-derived peptide P28, in the treatment of cancers, HIV/AIDS and malaria. Similar patent applications have been filed for the other promiscuous protein and peptide drugs by an Indian company Amrita Therapeutics (http://www.amritatherapeutics.com).

Will such bacterial protein or peptide drugs work in the real world? A company CDG Therapeutics (http://www.cdgti.com) in Chicago, under guidance from the US FDA, is evaluating the toxicity and side-effects of a chemically synthesized 28 amino acid peptide derived from azurin, called P28, in patients with advanced cancers and where no drugs are working. P28 not only proved to be non-toxic and non-immunogenic in animals but showed no side-effects, even at maximum tested concentrations, in such patients. Although efficacy is not evaluated in

phase I human clinical trials, the early results with P28 show interesting beneficial effects in such patients with advanced cancer where no other drug is working. Thus I believe that more such potential multi-disease-targeting drugs will be isolated and evaluated in the near future, clearly indicating that bacterial pathogenesis is not necessarily all evil, and something good can come out of it.

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The complete catalogue of life: an end or a mean?

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In the past 20 years, a multitude of new techniques have helped us peek into the cell at a scale and precision orders of magnitude higher than what have been able to observe until then. With the current rate of technological progress it will be possible in the next 20 years to accurately catalogue the parts list of almost every representative organism in the biosphere. In practical terms, this means we will have access to complete and correct genomes, transcriptomes, proteomes, metabolomes, interactomes – insert your favourite 'ome' here – for millions of different organisms. In other words, we will have 'captured the complete biome'.

While having a list of parts is of course a very useful, worthwhile and difficult goal, making sense of it is even more challenging. To do so, we need to have an accurate model of any cell state whether as a single entity or as part of a community, a tissue or a complex network of interconnected life entities. With the current computing technology this is not feasible, thus we are bound to seek short cuts and simplifications in our attempts to model life. We need to represent what is known about biological processes into retrievable and computable data objects – aka annotations. This is where biocuration is crucial.

Biocuration, which can be described as the translation and integration of biological information into an organized form such as a database, aims to facilitate the analysis of complex biological data. Biocurators and bioprogrammers are exploring techniques to help with data extraction and data representation. This area of research stands at the boundary between experimental research, informatics, statistics and even philosophy. The International Society for Biocuration (http://biocurator.org) has been created in 2009 to promote exchanges between experts in this rapidly moving area.

The road is long before we will have captured the earth biome. To do that we have to insure that the foundations of the corpus of biocuration that will be necessary to model life processes is robust. This can only be achieved by a collective effort that aims to overcome three main roadblocks in our progress towards translating genomic data into practical applications:

i. Models for data representation are poor and with few dimensions. Biologists have traditionally used hierarchical lists of terms as a way to represent biological entities. In recent years there has been much improvement in the representation of data by using much more formal descriptions of biological entities using ontologies. In an ontology, each entity is defined with relationships to other terms in the ontology. Many different types of relationships can be captured (*is_a*, *part_of*, *develops_from*, etc.). The advantage of this type of description for modelling biology is that it allows computing not only across terms, but also on how terms relate to each other. There are several ontologies used by biologists, the most widely used one being the Gene Ontology (Gene Ontology Consortium, 2010). Ontologies are a part of the wider 'semantic web technologies' (http://semanticweb.org) that allow different types of data to be represented in a consistent and compatible manner. The next challenge is in relating all those lexicons covering different areas of the biological space to support more complex data analysis, to work towards multidimensional data representation and querying.
ii. It is still very difficult to extract information pertinent to the specific question we are trying to answer from all the 'noise' in the data. The information we have in databases has variable confidence levels. All the knowledge we have accumulated in biology is obtained through the interpretation of a measurement of some parameter: for example, an enzyme activity may be measured by the absorption of its substrate or product; the role of a gene in development is often measured by the phenotype of a mutation; etc. Without a complete understanding of the biological role of the protein, the results of some of those experiments can be misleading. For example, mutations in a transcription factor can have pleiotropic effects, some of which may be very downstream from the

biochemical role of the protein. In addition to different types of experiments having different degrees of remoteness from the event being interpreted, different experimental set-ups have different degrees of reliability. Right now very few databases have ways to capture the confidence of different experiments. Low-confidence annotations (especially from experiments with low-confidence read-outs; rather than low-confidence data) can be extremely valuable for 'gold panning' approaches, where some 'weak' information, especially when confirmed by other data (which can be weak as well), may be valuable to provide testable hypotheses. However, that involves designing tools that can triage the high-quality, high-confidence data when appropriate, and look through low-confidence data when there is other data supporting them. The hope is that this will result in bioinformatics tools becoming more successful at finding gold nuggets!

iii. There is still much that needs to be learned about the functioning of cells. This goes a long way into explaining why much of the efforts in biological research are focused on information collection. Experimental research is of course where this starts and ends: the information, encapsulated in databases is used to build models, which are used to build new hypotheses. We are still struggling with highly noisy high-throughput technologies. This means that we cannot use currently obtained experimental results without a significant overhead of statistical and methodological filtering. Getting a reliable knowledge signal in the background noise of the data is a tedious Sisyphean task. As soon as one manages to solve the problem for a given set of data, the technology has already moved forward and one needs to come up with new tools (a good example is the progressive move from microarrays to deep RNA sequencing for gene expression studies).

Ultimately, the goal is to more accurately and rapidly answer meaningful questions having a direct impact on medical, agricultural and biotechnological issues. The hope is that all this accumulated data will help reduce the lag time between identifying a problem – for example: an emerging crop infectious disease – and its solution – in this case it could be the identification of a resistance mechanism against this pathogen. The complete catalogue of life, when it will be available, will provide the means to address those questions.

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Towards intelligent vaccines: the VAC-CHIP

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Vaccines can be exploited to prevent infections and other diseases (e.g. cancer) and, as a result, benefit both vulnerable populations (individual physical and mental discomfort and loss of work are avoided, and transmission is reduced) and dependent economies (the cost of treatment and loss of productivity) (Taylor *et al*., 2009). They are also increasingly proposed as a therapeutic approach for existing diseases (e.g. infections, cancer, autoimmunity), either as a stand alone measure or in combination with standard treatments, and thereby offer alternative clinical solutions (Riley *et al*., 2008; Chen *et al*., 2009; Dougan and Dranoff, 2009; Garren, 2009; Trimble and Frazer, 2009; Wraith, 2009; Andersen *et al*., 2010; Röhn and Bachmann, 2010). Moreover, the use of vaccines for non-health-related issues, such as birth control, is being explored both in humans and animals (Naz *et al*., 2005; Hardy *et al*., 2006; Bowen, 2008; Fayrer-Hosken, 2008).

Vaccine development, use, efficacy and safety are, however, complicated by their intervention in a finely tuned and regulated immune system that, when perturbed, does not always respond in the desired manner. Specifically: immune responses to a given antigen may, *inter alia*, be too specific, too unspecific, too weak, too strong, too short-lasting, induce auto-immune/disregulated reactions, etc. It follows that it is not sufficient to stimulate a strong response, but is also important to fine-tune the elicited response according to the specific clinical needs. Stimulation of the wrong response pattern might even lead to severe consequences (Salemi and D'Amelio, 2010), as it was the case for the first vaccine candidates against the respiratory syncytial virus, which promoted more severe clinical forms of disease in vaccinees (Fulginiti *et al*., 1969). Thus, considerable effort has in recent years been invested in developing different antigenic structures, more effective immunomodulators as co-immunogens (Ebensen and Guzmán, 2008), new antigen delivery systems (Liniger *et al*., 2007; Roy and Noad, 2008; Skountzou and Kang, 2009; Chadwick *et al*., 2010) and systems for targeting antigens to specific immune compartments/tissues (Ghosh *et al*., 2006; Tacken *et al*., 2006; Weiner *et al*., 2010), etc., in order to modulate both the intensity and quality of the immune responses to vaccine candidate antigens. Though these efforts have considerably improved the art of vaccinology, basic problems and variations in immune responses

still exist. For example, in the case of multicomponent vaccines it is difficult to address the issue of immunodominance and ensure optimal responses to all antigens in the formulation. Another challenge of some multicomponent vaccines is the need for different components of the formulation to stimulate different effector mechanisms (e.g. induction of neutralizing antibodies against one component and cytotoxic T lymphocyte responses against another). Moreover, in addition to the vagaries of general immune responses to diverse antigenic stimulations, there is a substantial diversity of immune responses of different individuals to the same immunogen, qualitatively and quantitatively, and even a diversity of immune responses in the same individual, according to age/health/hormonal status, such that a vaccine working optimally in the majority of vaccinees may work poorly or adversely in others (Guy, 2010).

Given the diversities of antigens, antigen formulations, general immune responses and individual specific responses, what is needed are intelligent vaccine systems (IVS), which are able to make and deliver antigens in an appropriate manner over time, while continuously monitoring and adapting to the immune responses occurring. Current advances in medicine, immunology, vaccinology, biosensors, nanotechnologies and cybernetics should render this dream feasible.

Imagine the following: a retrievable or absorbable micro-capsule – the VAC-CHIP – is embedded into an appropriate tissue. The capsule consists of a vaccine production module, a vaccine export system, a system for collection of body fluids, a system for analysing a battery of immune system effectors and products, and/or proxies/signatures thereof, and a microcomputer controller programmed to (i) analyse in real time immune responses to individual antigens, (ii) direct the new production and delivery of antigen to the immune system (either stored or *de novo* synthesized antigens or nucleic acids encoding for the antigens of choice, which will also enable optimal post-translational modifications), as and when required to achieve an optimal predetermined immune status, (iii) send to the 'Vaccine Centre' (VC) all data, and warn of any unwanted responses (e.g. cytokine storm by therapeutic interventions), and (iv) shut down antigen production, once the desired immune status has been achieved (e.g. by shutting down antigen production and/or starting an immunization sub-program leading to the stimulation of Treg cells (Corthay, 2009; Josefowicz and Rudensky, 2009; Nizar *et al*., 2010; Sun *et al*., 2010), and notify the VC that the immunization has been completed. Ultimately, communication between the VAC-CHIP and the VC should be automatic and direct, in real-time, although initially would probably involve manual readings taken by the patient, via an intermediary monitor-transmitter – probably based on a smart phone – which could monitor signals emitted by the microcomputer of the IVS, transmit them to the VC, receive instructions from the VC and transmit these to the microcomputer.

Once the desired immune response has been achieved, the VAC-CHIP could either be removed, or left in place, if absorbable. In the latter case, it is possible to imagine two scenarios. If residual biological components represent no significant disadvantage *vis-à-vis* toxicity/immune responses/subsequent immunizations, and the capsule itself is made of material that remains intact for the immunization period but is reabsorbed soon thereafter, then no further action would be necessary. If, however, the VAC-CHIP components present a potential risk, it is possible to imagine two further elements: one would be a VC-controllable biodestruct module that would contain/synthesize enzymes to degrade any remaining biological components (antigens, DNA, immunomodulators, etc.), which would themselves auto-proteolyse upon instruction from the VC, and a VC-controllable capsule destruct system, involving release of a chemical that would render the capsule material degradable and absorbable.

The VAC-CHIP should enable sequential administration of poorly immunogenic and immunodominant antigens, thereby guaranteeing optimal responses to both, as well as sequential delivery of vaccine formulations aiming at the stimulation of different effector mechanisms (i.e. dynamic prime-boosting).

It is also possible to imagine IVS systems that measure antigens as analytes and are programmed to deliver specific antibodies in response to the specific readouts. In this case, the IVS could be used for passive immunization (by supplying preformed antibodies or by manufacturing recombinant antibodies) of neonates and other populations that are immunodeficient, immunocompromized or requiring antibody therapy (e.g. patients infected with rabies; at risk from developing septic shock; etc.). Other potential applications might include a multicomponent antibody-based IVS that would monitor cognate antigens in an infant infectious disease control system, and one comprehensively targeting regionally relevant toxins that would effect therapy of snake/scorpion/spider bites without the need to identify the toxin before treatment, etc. Indeed, the basic concept of a therapeutic implanted chip that imports, measures and interprets a body analyte and exports an appropriate dose of a therapeutic agent, could be exploited in many therapeutic scenarios, including chronic diseases like diabetes and other hormone deficiencies, cancer, etc.

Current developments leading to the identification of molecular signatures for vaccine efficacy, and the advent of systems biology-driven approaches to model responses to infection and/or vaccination (Pulendran,

2009; Querec *et al*., 2009), will pave the road to the rational selection of proxies/analytes for decision-making by the VC. Approaches based on IVS might find an initial application for therapeutic vaccines or prevention of diseases caused by intracellular pathogens, which require complex responses against multiple antigens in order to confer efficient protection (e.g. CMV, HIV, tuberculosis, chronic hepatitis). They might be also the technology of choice for poor responders to vaccination (e.g. elderly, newborns). Most importantly, IVS would constitute a novel building block of personalized medicine, in conjunction with the current unravelling of the genetic basis of host responses and susceptibility to infections (Sancho-Shimizu *et al*., 2007; Blackwell *et al*., 2009; Flores and Okhuysen, 2009; van de Vosse *et al*., 2009).

It is also worth noting that an implanted IVS, or its therapeutic counterpart, would facilitate compliance in prevention and therapy treatment programmes involving populations, such as infants, the elderly, psychiatric patients, etc., or in judicial situations, where compliance may be problematic.

Although IVS might now seem to be just a dream, the nanomaterials, miniaturization and microfluidics technology to develop them is advancing rapidly (Heller, 2006; Myers and Lee, 2008; Pumera and Escarpa, 2009; Lenshof and Laurell, 2010; Oita *et al*., 2010; Scheinberg *et al*., 2010), so perhaps the VAC-CHIP is not so far away after all.

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Energy, climate and environmental biotechnology

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Meeting the needs of global development while maintaining high environmental standards is a difficult balancing act. This is more true today than at any time in human history. China, India and Brazil, three of the five largest countries on the planet, account for almost 40% of the world's population and their economies are burgeoning. With this comes an enormous appetite for energy – and enormous environmental impact. The empirical relationship between economic growth and pollution is often described by an environmental Kuznets curve (EKC), named after the Nobel Prize-winning economist Simon Kuznets who observed that the relationship between inequality and per capita income followed an inverted U – inequality initially increases with increasing income up until a point where inequality begins to decrease with further increases in income. A similar pattern is seen in the relationship between pollution and economic prosperity. EKCs have been observed empirically for pollution from sulfur dioxide, nitrogen oxides, lead, pesticides, chlorofluorocarbons and sewage. Interestingly, this relationship has not been observed for carbon dioxide emissions or natural resource use. The mechanisms that cause the inflection in EKCs are not completely understood, but the introduction of novel technologies and legislation to drive environmental improvements in an increasingly prosperous society are usually cited as proximal reasons. Environmental biotechnology has an important role to play in breaking the link between economic growth and pollution by delivering novel approaches for pollution mitigation, and by supporting more benign energy generation and reductions in energy use.

There is already considerable research and development effort targeted at the production of renewable biofuels; however, over 80% of the world's total primary energy supply currently comes from fossil fuels. Even under policies to reduce CO_2 emissions the International Energy Agency estimates that fossil fuels will still meet around 70% of global energy needs by 2030. The reality therefore is that in the short to medium term we will continue to rely on fossil fuels to feed ever-increasing global energy demand as current infrastructure is replaced with infrastructure supporting more sustainable, renewable energy sources. What therefore is the role for environmental biotechnology in this context?

The flip side of energy production from fossil fuels is the emission of CO_2. In this respect not all fossil fuels are equal. For example, in relation to electricity production the US EPA has estimated that per kWh of energy produced methane generates 0.569 kg of CO_2, oil generates 0.881 kg of CO_2 and coal, 0.963 kg of CO_2. As we transition from fossil fuel dependence there is therefore a logic to converting coal and oil to methane. Geochemical evidence indicates that methanogenic degradation of crude oil and coal *in situ* in petroleum reservoirs and coal seams appears to be a common process. Stimulation of indigenous communities of methanogenic oil and coal degrading organisms therefore holds some potential for production of cleaner fossil energy. Indeed such processes are already being developed commercially and are showing considerable promise. This approach begins to look even more attractive given that currently achievable recovery of light crude oil from conventional petroleum reservoirs is around 35% and for heavy oil, which dominates the world's petroleum inventory, levels of recovery are even poorer. By contrast around 70% of gas in place is recoverable. Considerable technical hurdles have yet to be overcome before energy recovery from solid and liquid

fossil fuels as gas can become a practical solution for biological recovery of energy assets. There has been considerable interest in research in this area from major energy companies who have invested in the Hydrocarbon Recovery and Conversion wing of Synthetic Genomics (http://www.syntheticgenomics.com/what/hydrocarbonrecovery.html) and the Energy Biosciences Institute programme on Fossil Fuels Bioprocessing (http://www.energybiosciencesinstitute.org/index.php). In addition to these high profile examples several other research groups and companies are actively pursuing this goal. Major investment like this holds considerable promise for biologically assisted energy recovery becoming a reality. Nevertheless, biology alone cannot provide all of the answers and integration of expertise from microbial ecology, petroleum geology and reservoir engineering will be essential to fully realize this major environmental biotechnology.

Environmental biotechnology not only has application in energy recovery, but also in reducing energy use. This is particularly pertinent to the world's largest biotechnology – biological wastewater treatment. In the developed world around 1.5% of electricity consumption is used to treat wastewater. For OECD countries this equates to over 1×10^{11} kWh of electricity. Conventional activated sludge processes consume around 0.5 kWh of energy per m^3 of wastewater treated. Over half of this energy budget is used for aeration. It therefore follows that anaerobic processes can significantly reduce the energy and hence environmental costs of wastewater treatment. We are therefore poised to see a resurgence of interest in anaerobic wastewater treatment systems, both conventional anaerobic digestion where key targets will be establishment of systems that operate stably even at low temperatures, and systems that can more effectively treat low strength domestic wastewaters with low residual organic load. A place will inevitably remain for nitrifying aerobic systems and for novel combinations of aerobic and anaerobic systems to achieve high treatment standards at lower energy cost. Novel microbial electrochemical systems such as microbial fuel cells (MFCs) may also offer a route to a reduced energy footprint of biological wastewater treatment even if these do not generate a single Watt of usable electrical power, simply by virtue of the fact that they have the potential to greatly reduce forced aeration.

In the age of synthetic biology and the astounding steps that have been made recently in this field it is inevitable that environmental applications of bespoke organisms for energy recovery and pollution mitigation are being discussed. This is an exciting prospect. It is however worth reflecting on lessons learned from the construction of genetically engineered organisms for environmental applications such as pollutant degradation. This research produced a huge and invaluable body of fundamental knowledge on the mechanisms of pollutant degradation and regulation of specific catabolic pathways, but did not deliver practical solutions to environmental contamination. Specially constructed organisms may be invaluable in controlled monoculture settings and one might argue that with the exponential increase in the ability to dissect and reconstruct genomes and the sophisticated data analysis techniques of systems biology, that challenges which were not overcome in the past can now be surmounted. Understanding what makes a cell tick is one thing, understanding how it competes effectively in the complex milieu of the natural environment may be an altogether greater challenge.

Microbes and food, partners in sickness and in health

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Crystal balls usually only allow a glimpse of shapes and shadows that require interpretation. When I gaze into the future of food microbes in health and disease, I see a situation in which food microbiologists develop ever more sophisticated strategies for improving food production, food quality, food safety and food and health, only to be confronted by the twin spectres of 'Regulatory Agencies' and 'Consumers'. Why spectres? Regulatory Agencies are often constrained to operate legislation that may not always based on current scientific knowledge, while we are often told that Consumers are 'not ready to accept' scientific advances in food science, such as irradiation, or genetically modified foods. I will try to use the crystal ball to bring into focus two aspects of the relationship between food microbes and man in which we are making significant scientific advances, but where we need to make equivalent strides in applying this knowledge – food safety and food and health.

First, food safety; foodborne disease is the consequence of an interaction between a food, a microbe and the human host, in which the outcome is unpredictable. Different individuals consuming the same amounts of similar microbes may remain healthy, or become sick, or even die. Regulatory Agencies rely on risk assessment to ensure a safe food supply, and these assessments are largely based on the numbers of microbes present (usually defined to genus or species level), and can seem to be based on round numbers rather than scientific evidence. Existing regulatory limits of 100 *Listeria monocytogenes* per gram in certain foods seem to imply that 90 bacteria are safe, but 110 are unsafe! There is no doubt that regulators have had an enormous impact in reducing morbidity and mortality associated with food in developed societies, but the problem of foodborne

disease has not been solved. We may be reaching a point where more sophisticated strategies will have to be considered to reduce not only the risks associated with foodborne pathogens, but also to decrease the waste associated with unnecessary recalls. It is likely that a significant amount of food is destroyed every year that contravenes regulations, but does not really pose a threat to the consumer. Equally, it is entirely possible that food will cause illness and even death even though it conforms to regulatory standards. In the future, the level of threat to the consumer may well be defined not only by numbers present, but also by the genotype of the individual organism(s) in the food. Does the strain have the necessary virulence factors to cause disease, are those virulence factors functional? The crystal ball predicts the use of near real-time quantitative high-throughput microbial genotyping to define risk levels. High-throughput mRNA analysis may even be used to assess viability, or the level of stress adaptation of the microbes *in situ*. I foresee regulations that will 'permit' certain levels of some pathogens, but enforce zero tolerance on others of the same species depending on their genotype and thus their virulence potential. This is obviously a complex and challenging approach to ensuring food safety, but it is the logical consequence of our increased knowledge regarding the actual virulence potential of individual strains. In fact, this more sophisticated approach has already begun. We already discriminate microbes more precisely that we could have imagined only a few short years ago. Current regulations distinguish between *Escherichia coli* as indicator organisms, *E. coli* virotypes with the potential to cause mild foodborne disease, and verocytotoxic strains such as *E. coli* O157:H7, which are recognized to pose a serious threat to consumer health. This is already the forerunner of a regulatory framework based on genotype rather than genera or species.

And what does the crystal ball predict for the future of microbes in food and health? Current regulations state that food may not be used to prevent, treat or cure disease, but this is surely semantics. Nutritional diseases are specifically excluded, such as osteoporosis, beriberi, scurvy or rickets, all of which can be addressed by diet. So the regulations seem to suggest that while certain diseases can be prevented, treated or cured through specific food interventions, it is not permitted to extend this list beyond nutritional diseases. But why should regulations constrain us from using food containing a microbe or another functional ingredient, which has been proven to have a positive effect in alleviating suffering? If rigorous scientific evidence supports the efficacy of a food microbe or ingredient in preventing the onset of a disease or in treating an ongoing condition, why should it be prohibited? Through the crystal ball I see this attitude changing radically over the coming years, to the point where we will look back and wonder at regulations that prevented consumers from benefitting from the meticulous and exciting scientific research in the area of food and health. I see foods containing microbes with demonstrated benefits in the prevention or treatment of inflammatory, atopic and infectious diseases, and perhaps even in reducing the incidence of devastating diseases such as colon cancer. Looking even further ahead, I see consumers welcoming foods containing microbes, which have been genetically enhanced to improve their health promoting capabilities, but always in the context of an informed risk–benefit analysis.

Now the ball grows dim (improving battery life is someone else's problem), but the way forward has become crystal clear – food developed in partnership with regulatory agencies and consumers, which reduces the morbidity and mortality associated with foodborne infections and their sequelae, and which also provides scientifically proven health benefits by delivering a 'payload' of microbes designed by nature or by science to improve the human condition.

Opening a black box: how microbial ecology can inform global climate models

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The field of biology is undergoing a revolution and is becoming increasingly interdisciplinary. This interdisciplinarity is not simply in the context of sub-disciplines of biological sciences such as physiology, biochemistry and molecular biology but, more strikingly, among fields as disparate as biology, chemistry, physics, mathematics and engineering (Kalluri and Keller, 2010). The accessibility of recent technological and conceptual innovations such as genome sequencing, transcriptome and proteome profiling technologies, easy to use experimental kits, high-resolution imaging and robotics has spurred design and fruition of many ambitious molecular characterization experiments in the animal, plant and microbial worlds (Kalluri and Keller, 2010). The field of microbial ecology sees a tremendous increase in the use of these new technologies, especially next generation sequencing technologies to analyse complex microbial communities. These complex environmental sequencing data enable follow-up experiments to determine environmental RNA profiling measurements and environmental proteome analysis directly from complex microbial communities.

One of the bottlenecks in our current investigative methods is in efficient generation, deposition, integration, analysis and mining of large and diverse datasets. Cross-disciplinary collaborations between biologists, instrument scientists, computational modelling and statistical experts

are needed to address this bottleneck. Data size derived from new analytical tools such as sequencing is anticipated to further increase significantly during the next years. Biology as a discipline will rely on the creation of an integrated data storage and analysis tool, also referred as Knowledgebase. The US Department of Energy, Office of Science is developing a Systems Biology Knowledgebase, envisioned as an open cyberinfrastructure to integrate systems biology data, analytical software and computational modelling tools that will be freely available to the scientific community. This Knowledgebase will drive two classes of work: (i) experimental design and (ii) modelling and simulation (for more information see http://genomicscience.energy.gov/compbio/).

Understanding the impacts of climate change is one of the great challenges we are facing today. A warmer climate will impact both terrestrial and ocean ecosystems in ways we do not fully understand. And, there are important feedbacks to the climate system from these ecosystems as they warm and respond to higher concentrations of atmospheric CO_2. We need to understand the tightly coupled biogeochemical cycles (carbon, nitrogen, water, etc.) and the important drivers of those cycles that occur at all scales of biogeochemical organization (Schimel, 2004). At the largest scale of space and time are phenomena such as the 'Great Ocean Conveyor', which circulates water (plus chemicals and heat) through the oceans of the planet, spanning to the smallest scales of organization like chemical reactions in the atmosphere or biogeochemical dynamics in terrestrial ecosystems. Microbial processes dominate global biogeochemistry, accounting for roughly half of global photosynthesis and almost all organic matter decomposition, nitrification, denitrification and methane production (Schlesinger, 1997). Microbial processes, however, are regularly treated in models as a simplistic black box, although the details of microbial physiology can have large impacts on global biogeochemical cycles and the planet's climate system (Schimel, 2004).

The carbon and nitrogen cycles are especially critical to understanding future climate change and the response and feedbacks of terrestrial ecosystems to future changes (Thornton *et al.*, 2009). Terrestrial ecosystems that have been identified to be particularly important include tropical forests, boreal forests, peatlands and arctic permafrost. These ecosystems contain large quantities of carbon that if released to the atmosphere as CO_2 or CH_4 could provide significant positive feedback to the climate system and exacerbate global warming. Boreal forests, peatlands and permafrost hold a tremendous quantity of the Earth's buried soil carbon and are especially vulnerable by being in the high latitudes where warming will be highest. In permafrost, the seasonally thawing active layer comprises the top centimetres to meters of permafrost soil and

Fig. 1. A prototype warming chamber constructed at Oak Ridge National Laboratory that is being tested for refinement before deployment for an ecosystem manipulation experiment in a spruce – peat bog near Grand Rapids, MN, USA (https://mnspruce.ornl.gov/). Photo compliments of Paul Hanson, Oak Ridge National Laboratory.

accounts for most of the tundra's biological activity and Green House Gas (GHG) releases. Current climate models predict a significant increase in thawing depths and durations of thawing, turning permafrost land into wetlands or thermokarsts where microorganisms can mineralize complex organic matter and release CO_2 and CH_4 as well as N_2O and other GHG (Zimov *et al.*, 2006; Elberling *et al.*, 2010). Models simulating current GHG production and transport do not yet adequately address permafrost thawing as a result of global warming, changes to above-ground vegetation, or microbial mineralization of buried carbon.

Climate change scientists have conducted field experiments, often at large scale, to see how ecosystems will respond to more or less precipitation, rising concentrations of atmospheric carbon dioxide and warming temperatures. Experimental data are key to determining if and to what extent ecosystems will be affected by climate change in 50 to 100 years and how those changes might feed back to further advance change (Wullschleger and Strahl, 2010).

Two next-generation ecosystem experiments are being planned for deployment that will shed light on these important processes. An experiment to expose a boreal forest – peat bog ecosystem in Grand Rapids, MN, USA to both warmer temperatures and elevated CO_2 concentrations is under development using an innovative combination of belowground and aboveground warming technology (see Fig. 1) while also enriching the CO_2 concentration in the atmosphere around the forest (Hanson *et al.*, 2010). A second experiment is being considered for implementation in the permafrost in Alaska, USA (S.D. Wullschleger, pers. comm.). Both of these experi-

ments will provide critical large-scale process understanding to improve earth system models. But, they also offer unique opportunities for microbiologists to collaborate with plant biologists, ecologists and geologists. The scientific opportunities to explore biogeochemical cycles at multiple scales with modern biological, chemical and physical methods is unprecedented and will challenge us to develop new informatic tools to integrate these new data that span disciplines and scales. With these integrative studies we may begin to open up the proverbial black box and quantify important interfacial and molecular biogeochemical processes.

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Metabolic engineering, systems biology and synthetic biology

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In the sunny morning of November 15, 2017, Professor Meteng is having a conversation with one of his graduate students in the lab. 'Have a look at this. I just finished designing the 3.5 kb bacterial genome for polyethylene terephthalate (PET) production. Wow, it took us 2 weeks to design this sequence using our *in silico* MetabolicRegulatorySignaling Designer program. As we discussed, we now optimally positioned all the genes necessary for cell growth and PET production from carbon dioxide and sunlight. We should be able to produce PET with a productivity of 5.5 g l^{-1} h^{-1} and 95% of the maximum theoretical yield. We still need to run several fermentations to confirm the *in silico* predicted performance and collect data in order to further improve this bug's performance by fixing its genome. Once these are all done, I expect that the production cost of this bio-based PET will be about 2.5$ per kg. Later today, I will send this synthetic genome sequence to the National Genetically Created Organisms Committee for approval to synthesize. It usually takes five working days to get approval. Then, I will send the sequence to company X to synthesize it. The synthetic genome will cost $500 and will be delivered to you within 24 h. We might get some discount as we already synthesized two genomes last week. Once you receive the synthesized genome, you can create PET producer using the *in vitro* CELL CREATOR kit we purchased last week. How do we want to name this bacterium? Hmm . . . how about *Plasticomonas superproduciens* KAIST2017?'

Over the last couple of decades, metabolic engineering (Bailey, 1991) has advanced rapidly to design and develop engineered bacteria in order to more efficiently produce drugs, chemicals, fuel and materials. Traditional metabolic engineering started by manipulation of a handful of genes to be amplified, deleted, and/or introduced heterologously. Over the last decade, advances in systems biology have changed the way metabolic engineering is performed. Based on the rapid analysis of the entire genome followed by other omics studies including transcroptomics, proteomics, metabolomics and even fluxomics, metabolic engineers are now equipped with vast amounts of data and simulation tools that can be used in designing the optimally performing cells. A system-wide analysis and engineering of metabolic and other cellular networks are now possible – and thus called, systems metabolic engineering (Lee *et al*., 2007). More recent advances in cost-effective DNA synthesis and synthetic biology are triggering our interest in possibly designing the whole genome followed by actual genome synthesis. Although Craig Venter's the first artificially synthesized genome (Gibson *et al*., 2010) is far away from the creation of true 'designed cell doing something useful', it showed the possibility for the first time.

Are we really interested in creating artificial organisms, such as overproducers of biofuel, plastics, chemicals and drugs? The answer is YES; but first, we might want to define what artificial organisms are. If we start with our favourite microbe *E. coli*, and engineer it by knocking out 50 genes, amplifying 10 genes, altering 7 regulatory circuits, and introducing 10 heterologous genes originated from plants and animals, is the resulting strain an engineered *E. coli* or created artificial cell? What if the number of genes that are altered reaches over 50% of the original genome? What do we call it? One thing for sure is that this

type of engineering (or creation) should be performed for the benefits of human and environment. Ethics, safety and security issues will become increasingly important (Lee, 2010).

Metabolic engineering is an essential paradigm for the environmentally friendly production of chemicals and materials from renewable resources with an aim to save our earth and sustain human race, and developing ways of efficiently producing drugs that are new or difficult to synthesize. Innovative ways of bioremediation using metabolically engineered organisms will also be developed. Looking ahead, it is likely that many sophisticated organisms developed by systems metabolic engineering will appear over the next 7 years, mainly for the enhanced production of drugs, chemicals, fuels and materials. Some of these will be incorporated into the actual industrial biorefinery processes for the mass production. At the same time, organisms that are based on 100% synthesized genomes will continuously appear; the first handful of these will be most likely mimicking the genomes of the organisms already present in nature. We should not think that we are playing God.

It is also likely that systems metabolic engineering approaches will be adapted to new therapeutics and disease prevention. Human body (and other organisms as well) is a truly complex system, which will almost never be completely understood. Our current therapeutic paradigm is a single drug-single target approach, while multiple drugs (a mixture of single drugs) can be administered to treat multiple respective symptoms. An old Korean saying 'the best drug is good food' deserves good attention. The traditional Korean medicine book 'Dong-Eui-Bo-Gam' written by Hur Jun 400 years describes many interesting therapeutic recipes for treating chronic diseases. Obviously, millions of secondary metabolites among others present in these mixed plant extracts cannot be considered as drugs in the current drug administration standards. If multicomponent multi-target interactions are clarified at the systems level, and designer plant combinations without side-effects can be formulated, it will change the human healthcare paradigm – to this end, systems metabolic engineering will play an important role. Ultimately, what we eat (food) will be systematically coupled to our health issues through systems nutrition, and for this, the approaches developed in systems metabolic engineering field will become essential.

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Bioprocess Systems Engineering: bridging the 'scales' between 'molecules, cells & processes'

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Mathematical models of biological systems developed over the last decades incorporate various degrees of structure and mathematical complexity. Focusing on microbial systems, models of single cells, cell populations and cultures have been central in the understanding and improvement of the cellular systems, as well as in the optimization and control of the bioprocesses (Thilakavathi *et al*., 2007). In the last decade especially, the large-scale generation of biological data obtained with the development of a variety of high-throughput experimental technologies enabling system-level measurements, demand for mathematical model building to become a centre of importance in biology (Covert *et al*., 2001). Alas, as Bailey (1998) argued the development of mathematically and computationally orientated research has failed to catch up with the recent developments in biology. The need to bridge this widening gap between mathematically oriented research and system-wide analytical experimentation ('omics' technologies) stimulated *Systems Biology* to emerge as a new and dominant science (Bruggeman and Westerhoff, 2007). Consequently, the logical question is being raised of whether *Systems Biology* has simply rushed into the development of new biological theories in the quest to convert data into knowledge? In a field now shifting from method development to application development (Oberhardt *et al*., 2009), there is now some increasing scepticism regarding the future of *Systems Biology* as understanding of all the collected information has lagged far behind its accumulation because modelling of complex systems is an inverse problem that cannot be solved (Brenner, 2010).

Simply put, even relatively simple microorganisms, which have been extensively studied, are hosts to a complex network of interconnected processes occurring on diverse time scales within a confined volume. The multilevel nature of the regulatory network and the interactions occurring at the intracellular level further augment this complexity (Yokobayashi *et al*., 2003). Therefore, attempts to wholly model the function of even a single cell are non-trivial, if not impossible. The amount of delicate intracellular measurements required to validate such a

model is exhaustive both in terms of labour as well as cost, even with the arsenal of the 'omics' technologies at hand. Furthermore, uncertainties introduced on the parameter identifiability level (Sidoli *et al*., 2003) and on the mechanistic level further complicate this task. The balancing point for the trade-off between fidelity and tractability is a constant concern even with the advancements in both numerical tools and raw computational power. A thorough overview of the literature reveals that the optimal point on the scale between tractability and fidelity does not lie near the boundaries. What ultimately discriminates a good model from a bad model is its ability to successfully describe the modelled process while minimizing the uncertainty of its output variables. However, in the majority of studies the most common approaches followed are either utilization of literature data to validate the models or generation of experimental data without any form of systematic design of experiments. From an engineering point of view, this information can only be utilized through a systematic and rigorous framework that will organize and prioritize necessary measurements and experiments while simultaneously maximizing information obtainable from the data (Sidoli *et al*. 2004).

The ever-increasing development and improvement of sustainable bioprocesses towards efficient production of pharmaceuticals and chemicals inadvertedly requires the implementation of a rational and feasible approach. Process systems engineering offers a variety of modelling, simulation and process evaluation tools, which are widely used in the chemicals and fuels sectors where even small process improvements may bring substantial economic profits (Jimenez-Gonzalez and Woodley, 2010). Therefore, the need for a *Bioprocess Systems Engineering* research framework may prove beneficial. We have formalized such a systematic approach for modelling biological systems (Kontoravdi *et al*., 2005; 2007; 2010; Kiparissides *et al*., 2009; Koutinas *et al*., 2010), shown in Fig. 2. In our opinion, a mathematical representation of a studied biological system should aim to describe the system using adequate yet not excessive information regarding the relevant biological mechanisms. Overly complex mathematical formulations that lead to over-parameterized models should be avoided. Rigorous model analysis is required to screen for significant and/or redundant parameters and design optimally informative experiments in order to refine the parameter values and minimize the uncertainty in the model output even potentially leading to model reduction. The aim should be a 'high-fidelity' model that is successful over a wide range of operating conditions that can be used for model based optimization and control. Maybe then we can successfully bridge 'scales' between 'molecules, cells & processes' that will render modelling practically useful to biology.

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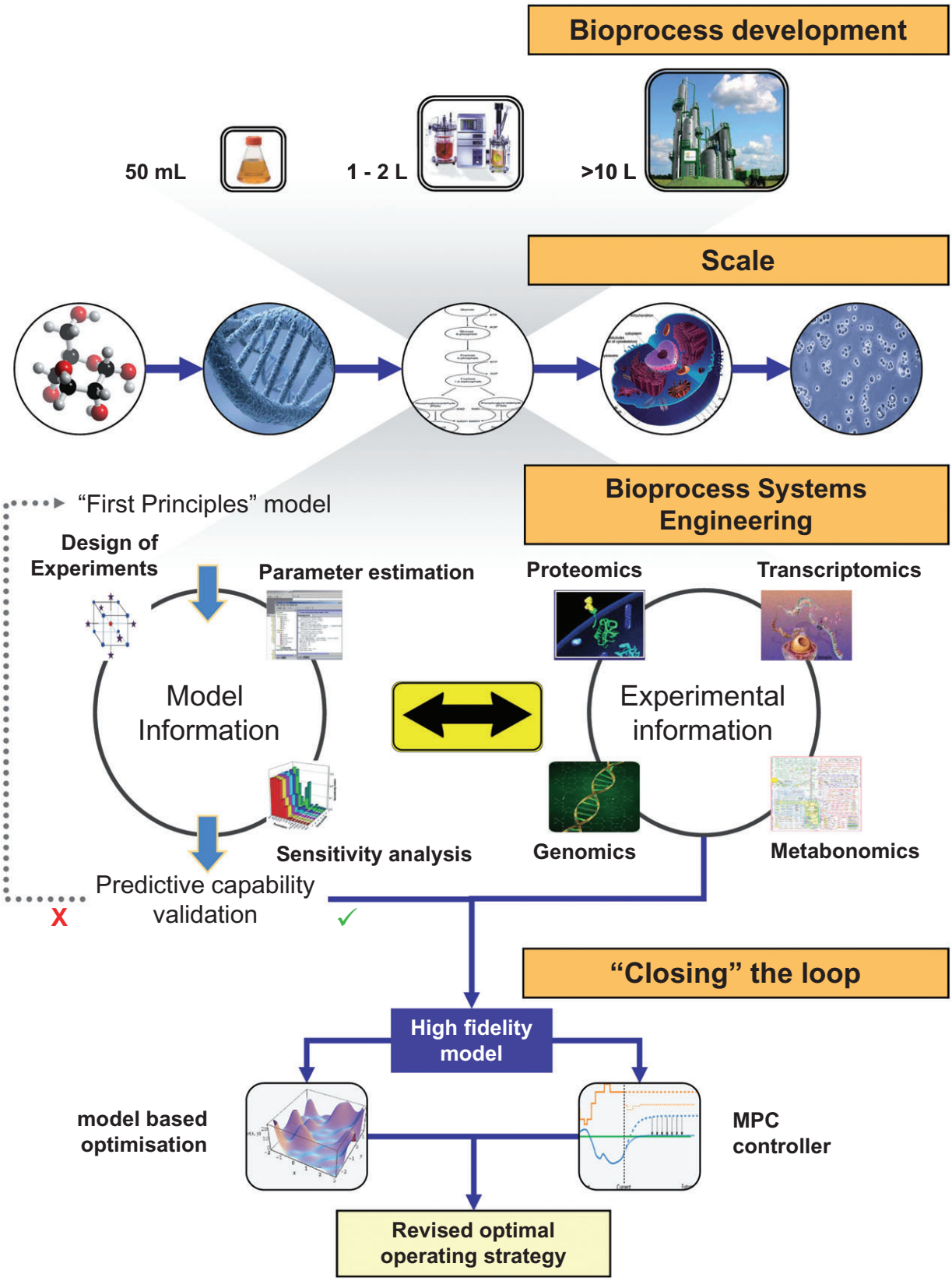

Fig. 2. Model development framework for bioprocess systems engineering.

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Microbes watching over us

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Foretelling the future is a rare gift privileged to very few among us. Luckily, some things appear so crystal clear that even a lay oracle as myself can make reasonable guesses as to their future developments. For the sake of this crystal ball worth concentrating upon is the foreseeable lack of clean primary resources and the need for increased resource recycling and monitoring in an ever-increasing demanding and populated society. In particular the availability of clean water is of growing concern, given the wide variety of direct and indirect water usages (Gleick and Palaniappan, 2010). Although water is in principle a renewable resource at a global scale, strong regional differences of water use and availability (flux) lead to non-renewable and unsustainable use (Gleick and Palaniappan, 2010). In addition, used water streams are often (severely) contaminated, and this precludes recycling on a regional scale unless appropriate physicochemical and or biological treatment systems are installed. Our discipline can praise itself for its past accomplishments to understand, develop and apply microbial activity-based processes that help recycling organic waste streams, purify water, recover nutrients or improve hygienic aspects of water supply. Unfortunately, many existing effective treatment processes (e.g. biological wastewater treatment) are not implemented in large areas of the World, exacerbating the degradation of water quality in those areas and, as a consequence, depleting local water sources and fluxes. Even without new future process developments, therefore, much could already be improved by implementing existing microbiological treatment processes.

Constant recycling of used water at a small regional scale, as, for example, typical in European river basins, not only demands excellent purification and treatment technology, but also a rigorous implementation of quality monitoring, both at the 'source' and the 'waste' level. This is typically done by well-equipped analytical labs, but even so, constantly appearing new compounds of toxicological concern (e.g. pharmaceuticals) demand continued efforts in analytical developments. Other areas of the World are characterized by decentralized water supplies, even at the level of individual households (Fendorf *et al*., 2010), in which case quality control is not trivial, not even for compounds that we consider 'standard' in western countries (e.g. heavy metals). In order, therefore, to increase the potential for water recycling at a regional scale, appropriate purification technologies and effective monitoring methods have to go together. So what can microbial biotechnology contribute to those aspects? Here I would only like to concentrate on the 'monitoring' aspect, which has seen a steady development of the concept of bioreporter assays over the past 15 years or so (van der Meer and Belkin, 2010). Bioreporter assays implement living cells (prokaryotes, yeasts, other types of cell lines) as analytical tools. The idea behind bioreporter assays is to exploit the capacity of every cell to sense the presence of chemical compounds in and outside the cellular compartment. Chemical sensing by cells is performed by membrane receptors with specific ligand-binding domains or by cytoplasmic regulatory proteins, that can relay effector binding to *de novo* gene expression. In bioreporter cells, the cognate signal cascade is replaced by a gene circuit in which activation of the sensory protein results in expression of a so-called reporter gene, such as luciferase or an autofluorescent protein. Use of such non-native reporter proteins enables to measure reactions of cells very sensitively, non-invasively and at low concentrations of chemical compounds. Bioreporter technology can thus be used to produce cheap and robust biological assays to measure the presence of a variety of chemical compounds. Presently, two categories of systems have been produced; those, which target specific chemical compound classes, and, second, those, which target any compound that exerts 'toxicity' or 'stress' to the cell (van der Meer and Belkin, 2010). Bioreporter assays come in various assays, and currently permit easy and sensitive measurement of compounds not only in aqueous samples, but also food stuffs, other solid materials, biological fluids or in the gas phase. Bioreporter assays have been extensively tested in numerous different laboratories, in environmental settings and in comparison with chemical analytics, and this has demonstrated that the technology is 'ripe' and can be applied at various levels of complexity.

What is then my wish list for future developments in this area? I hope that the technology will reach a 'legal' breakthrough in the sense of becoming recognized as official test in OECD procedures. This would help enormously in implementing bioreporter tests to standard analytical labs and propose them as realistic alternatives in areas where chemical analytics is too expensive or unavailable. Bioreporter engineering is also one of the very concrete applications of synthetic biology, where gene circuits with different biological parts are assembled into workable bioreporter strains. There are many examples of how more and better parts would be useful for bioreporter technology. For example, it would be extremely valuable

to have more sensory proteins with different chemical recognition specificities. This will require efforts to combine protein structure information, genome searches for homologous proteins, and directed evolution approaches, in order to more reliably design sensory proteins 'à la carte'. Synthetic biology will also help to understand how to build new types of reporter circuits with signal amplification procedures or reduced background. Finally, bioreporter technology will profit enormously from a true integration with micro-engineering (van der Meer and Belkin, 2010). Because of their small size, bacterial and yeast reporter cells can be easily integrated on microfluidics platforms, where the cells can potentially be cultured and their signal followed continuously. An integration into microfluidics platforms may also permit the development of true reporter arrays, by which a multitude of strains equipped with different sensing specificities can be confined each to an individual small area on one platform. This would enable measuring multiple target compounds simultaneously.

Microbes watching over the quality of our primary resources; who would have thought this?

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Model guided engineering of microbial metabolism

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In recent years there has been a rapid growth in the development of genome-scale metabolic models for different microorganisms. These models are traditionally reconstructed through a bottom-up approach, where genomic information is combined with information from databases and the literature to establish a list of biochemical reactions that can take place in the organism under study. The resulting metabolic network is then undergoing further analysis, where gaps in the different metabolic pathways are filled, either through further search in the genome or simply by inferring the specific enzymatic function that will ensure gap closure. The resulting, gap-free, metabolic network can then be used for simulation of metabolic functions through the use of flux balance analysis. This allows for rapid evaluation of the metabolic capabilities of the microorganism studied, and this allows for evaluation of different metabolic engineering strategies, e.g. what is the impact of deleting specific genes and what is the impact of inserting specific genes on the microorganisms ability to produce a desirable metabolite. There have already been described several successful application of genome-scale metabolic models for identification of metabolic engineering targets, and clearly the future will bring many further examples.

However, to further advance the use of genome-scale metabolic models in metabolic engineering two things will be required: (i) a faster route for constructing and/or updating these models and (ii) better description of metabolic regulation. The first will require better integration of model building/revision with databases, such that new annotations can rapidly be integrated into the models and hence improve their performance. This will require bridging between genomics, bioinformatics and metabolic modelling. The second will require integration of regulatory information into genome-scale metabolic models. Even though there is progress on incorporation of thermodynamics into these models, and some initial work has been done on including regulation in a few models, still much more is needed to advance the predictive strength of genome-scale metabolic models. However, I am certain that within the next 3–5 years we will have seen substantial progress in this field, and will be presented with metabolic models that are better integrated with genomics databases and that incorporate extensive regulation. This progress will be pioneered by work on the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae*, which besides serving as model organisms also serves as important industrial cell factories.

Microbial ecology and water engineering

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We have a 'Specialist Group' in the International Water Association named 'microbial ecology and water engineering' and I have borrowed this title for my look into the crystal ball. Both because the topic is extremely important for the big challenges facing the rapidly growing human population in treating wastewater and providing drinking water – but more importantly because a paradigm shift is currently underway in our understanding of microbial communities in the context of water engineering and thus Environmental or Microbial Biotechnology. When I look in the crystal ball I'm,

however, not sure whether it is mainly a 'I believe' or 'I hope' – but perhaps it is both as described below.

The future of microbial ecology will provide many exciting things to the engineers and – what many tend to forget – also visa versa: New microbial processes have in the past been discovered in engineered systems, e.g. the process for removal of the nitrogen by anaerobic ammonium oxidation (anammox) and enhanced biological removal of phosphorus. In case of the anammox organisms, they proved not just to be important in engineered systems, but central to the whole global N-cycle. The next decade will undoubtedly reveal several new organisms or microbial consortia that can be developed into useful full-scale processes in water and wastewater treatment or in the recovery and reuse of non-renewable recourses, such as phosphorus and production of bioplastic.

In order to manage the microbial ecosystems, microbial ecology needs to provide engineers with better models and theories about how these complex ecosystems work. Many wastewater treatment plants are still run as a 'black box' with a minimum of knowledge about the functioning ecosystem that lies behind. In the not too distant future I see the development of representative 'model ecosystems' where a detailed understanding of the underlying organization of the ecosystem has been obtained. Bill Sloan and Tom Curtis have initiated to incorporate the huge microbial diversity into relatively simple models that take the first steps at elucidating the general rules for the assembly of microbial ecosystems (Ofitery *et al*., 2010). I see a further development of such 'top down' models and a meeting of these with the deterministic 'bottom up' metabolic models and mass transfer models, e.g. by advanced 3-D biofilm models as developed by Cristian Picioreanu and Mark van Loosdrecht (Graf von der Schulenburg *et al*., 2009). It will be interesting to see these approaches merge in the future.

Metagenomics and other '–omics' methods are extremely useful to obtain lots of information about specific ecosystems. The number of studies of engineered systems – although is still few – have given invaluable new knowledge, but have mainly been limited to the sequencing of genomes of uncultured organisms such as the anammox bacteria, polyphosphate-accumulating organisms and nitrifiers obtained from highly enriched cultures. Metagenomes of entire model ecosystems will characterize the diversity and potential function of the entire community and are now becoming practically possible because of rapidly improving sequencing and bioinformatics capacity. A deeper understanding of ecosystems must combine these approaches with transformation rates to characterize the overall function of the system. These advances will be combined with advances in single cell techniques that link the genomic information with species-related morphology, surface properties, 3-D organization in the aggregates and other functional details. The combination of '-omics' with single-cell studies is extremely important – just ask a plant operator where the entire treatment plant is covered by a thick foam layer caused by a certain filamentous bacterium excreting hydrophobic surface components! My crystal ball sees these studies eventually developing an easy and fast community fingerprint of structure and function that contains both a diagnosis of problems and suggestions for corrective actions of relevance for the operation of the system.

Is the microbial diversity in engineered systems so high that it is beyond our reach to cope with? Fortunately, not so! We have been studying in a large number of Danish wastewater treatment plants with enhanced biological phosphorus removal and discovered a surprising similar composition of the microbial communities (Nielsen *et al*., 2010). However, indications are strong that the microdiversity among the different species or genera is very large in the individual plants and perhaps partly decisive for plant function and stability – which should be resolved in near future.

The exciting new microbial ecology and 'Systems Biology' will go hand in hand with the next generation of ecosystem theories and modelling and – not to forget – the developing of new technologies in water engineering. This will require more co-ordination and cross-disciplinary collaboration – already at the university and during post-graduate training. Strangely, this aspect of the future is – in my crystal ball – still unclear. I certainly hope that this wish will also come true.

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Switch on the bugs! (Where Environmental Engineering meets Synthetic Biology)

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Microbial respiration is a form of internal electricity generation. In 1962 Davis and Yarbrough elegantly exploited this in the first true MFC: electrical power was produced

from organic conversion and sunlight (Davis and Yarbrough, 1962). In 1979 the process was reversed, electricity was given to a fermenting organism and its product spectrum changed (Hongo and Iwahara, 1979). While MFCs resurfaced strongly in the past years, we now know that effective power production at scale is challenging, and that the economic and environmental drivers for this bioelectricity are limited. Aside from developing advanced bioremediation approaches, many researchers are now refocusing towards the production of chemicals and fuels with this technology ('bioelectrochemical systems'). Electrical current can indeed be used to drive microbial metabolism for bioproduction, by changing the outcome of fermentation, or by driving respiration ('microbial electrosynthesis') (Nevin *et al*., 2010; Rabaey and Rozendal, 2010).

Producing (bio)chemicals starting from electricity and CO_2 or substrate organics could have many advantages, such as high-production density, CO_2 capture, facilitated storage and 'transport' of electricity. Particularly attractive is the combination with wastewater. Although its supply is limited and localized, wastewater organics can provide an energy-efficient source of the electrical current and CO_2 (anode), while the wastewater nutrients can support the growth of producing organisms (present at the cathode). Principally, microbial electrosynthesis uses microorganisms to convert mixed organics into electrical current, and converts electrical current into specific organics of interest. Biorefining in one reactor!

What is needed to make this work is a good technology platform (including electrode materials), microbial populations to degrade the wastewater organics and effective production strains to convert the electrical current into the desired product. We have none of these today. This is hence where environmental engineering meets synthetic biology. Breakthroughs are needed to create biocompatible, low-cost electrode materials, combining surface area with surface charge and functionality. We need to engineer microorganisms that effectively produce biochemicals using electrical current as electron donor. And, we need to develop a technology that brings this process to scale, in a wastewater context or in a straight bioproduction context. Quite some walls between microbiologists and engineers need to be torn down, and the challenging creation of a common language will be part of this.

If we succeed, we will extract sufficient value from wastewater to turn it from a waste into a resource, with a positive value. Wastewater treatment can thus be professionalized to the level of true biorefining, where water, organics/energy and nutrients are extracted and brought back to the market. Outside the wastewater context, the sun can (via photovoltaics) drive microbial electrosynthesis, and thus enable bioproduction in areas that are not suitable for agriculture. Areas such as the Australian outback and the Arizona desert suddenly provide a tremendous opportunity for large-scale biorefining. So if all goes well, you may find that someday your wastewater will go to the highest bidder, and that you can fuel your car not only in the Saudi-Arabian desert . . .

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Giving a little help to our prokaryote friends

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Because of their small size and simple physiology, prokaryotes are especially capable of exploiting an amazingly wide range of metabolic niches that also provide wonderful services to society. I illustrate the amazing variety by giving a few examples of prokaryotic physiologies that are relatively recent discoveries and that hold promise for providing important services:

- Certain bacteria are able to carry out respiration via extracellular electron transport through a conductive biofilm matrix (Torres *et al*., 2010). These anode-respiring bacteria offer promise for producing renewable electricity, hydrogen gas and other materials from biomass organics.
- Species of *Dehalococcoides* are able to reductively dechlorinate organic solvents, such as trichloroethene, to harmless end-products in another unique form of respiration (Löffler *et al*., 2005). *Dehalococcoides* already are being used commercially for bioremediation of solvent-contaminated sites.
- The ANAMMOX planktomycete is able to oxidize ammonium while respiring nitrite, creating a novel form of anaerobic ammonium oxidation (Strous *et al*., 1999). This startling discovery is being pursued for the treatment of high-ammonium waste streams (Van Loosdrecht *et al*., 2004).

High-value services for pollution abatement and renewable energy/materials also can be obtained by exploiting 'mundane' microorganisms, such as methanogenic *Archaea* to produce renewable methane (Speece, 2008), ammonia- and nitrite-oxidizing bacteria to eliminate ammonium pollution (Siripong and Rittmann, 2007), and hydrogen-oxidizing autotrophs to reduce nitrate, perchlorate, and a range of other oxidized contaminants (Rittmann, 2007).

Whether the microorganisms are novel or mundane, sometimes they need a 'bit of help' so that they can sustain themselves as they provide society with its service. This help can take different forms – biological, physical or chemical. Examples of help are increasing their metabolic capability, making their substrates more available, protecting them from toxicity, and retaining them in a favourable location. Cleverly applying the right kind of help will become more and more important as environmental biotechnologists strive to gain more services from the microbial communities.

One biological approach involves modestly redirecting the metabolism of a well-suited microorganism so that it provides the service that society desires. This approach is sometimes called metabolic engineering (Stephanopoulos *et al*., 1998), and it differs from 'synthetic biology' approaches that attempt to construct cells *de novo* (Chopra and Kamma, 2006). The likelihood of success is greatly increased when the added metabolic functions expand the capability of a microorganism that flourishes in the conditions required to provide the service. My colleagues Willem Vermaas and Roy Curtiss are taking this approach to direct the metabolism of the cyanobacterium *Synechocystis* PCC6803 to produce and excrete long-chain fatty acids that are harvested directly from the reactor medium; the fatty acids then become a renewable feedstock to make liquid transportation fuels. This approach exploits a robust phototrophic bacterium and eliminates the need to harvest biomass and extract lipids from the biomass. Vermaas and Curtiss achieve their goal by adding genes for enzymes that produce the fatty acids, while also deleting genes for enzymes that consume the fatty acids. If ultimately successful, this metabolic help will allow PCC6803 to absorb sunlight energy and fix CO_2 into a renewable petroleum replacement, making PCC6803 a true photosynthetic factory. Thus, PCC603 remains a phototroph, and we help it produce our desired product, long-chain fatty acids.

A form of physical help that has widespread applicability for generating renewable energy from biomass involves pretreating the biomass to make it more bioavailable for conversion to methane, hydrogen, or other energy forms by anaerobic microbial communities (Rittmann, 2008). Several biomass-treatment technologies are now commercially available to disrupt biomass aggregates and cells to make them more bioavailable (Salerno *et al*., 2009): e.g. mechanical shearing, ultrasound, heating, freezing, and exposure to pulsed electric fields. The anaerobic communities still ferment and respire in ways that produce valuable energy outputs, such as methane or hydrogen gases, but pretreatment helps them do it faster and more completely (Rittmann *et al*., 2008; Salerno *et al*., 2009).

Providing our prokaryote friends with a bit of help follows the basic principle of environmental biotechnology (Rittmann, 2006): 'We work for the microorganisms so that they work for us.'

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Bacteria and nanoscale biophysics

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Small is beautiful. During the last years we have witnessed a spectacular breakthrough in the design and fabrication of tailor made artificial templates or metamaterials at the nanoscale with outstanding properties. This is a direct product of the nanotechnology revolution that has permitted superior performance first on electronic devices, microelectromechanical systems, and more recently in the manipulation and confinement of light. The main idea behind structuring materials down to dimensions matching the characteristic sizes of the elementary blocks (i.e. electrons in metals, photons and plasmons in lights, or fluxons in superconductors) is that boundary conditions become more relevant and lead to pronounced modifications of the general response of the system. In physics, the most popular example is the emergence of the discrete character of nature, the so-called quantum limit, once we reach this nanoscale realm.

The curiosity for exploring the effects of spatial confinement and the importance of the scale is currently also taking a leading role in the world of microbes. This was already anticipated in the previous Crystal ball edition back in 2007 by Les Dethlefsen and Relman (2007). This novel research line lies at the crossroads of several disciplines such as nanoengineering of microfluids, lab-on-a-chip technology, modern nanofabrication techniques, chemistry, physics and microbiology. Therefore, to guarantee a successful development of this colossal task, it is imperative to achieve a mature scientific synergy among this rich diversity of fields.

Nowadays, in almost every modestly equipped laboratory across the world, submicrometer patterning of materials that allows one to easily prepare small habitats matching the dimensions of bacteria can be routinely achieved. This has permitted Männik and co-workers (Männik *et al*., 2009) to investigate the effects of submicron constrictions on the motility of bacteria revealing their previously unexpected skills to survive and proliferate in this environment at expenses of shape adaptation. Interestingly, a major difference in shape adaptation of *Escherichia coli* and *Bacillus subtilis* is observed, likely related to their differences in cell wall architecture. Scanning probe microscopy techniques providing information about the nanoscale surface architecture of living cells can reveal what exactly is going on at the cell surface (Dupres *et al*., 2010). So far, it is not known how this information of spatial confinement is processed by the bacteria. Is the adaptation the result of physiology or mutation? Understanding the adaptation of bacteria to such extreme situations might have a direct and immediate impact on soil microbiology, behaviour of bacteria in tissues and biofilms, water filtering systems and biomedical research. At these scales, not only the dimensions of the constrictions become relevant but also their particular shape. For instance, asymmetric funnel shaped barriers can lead to rectification or biased bacterial motion permitting one to control their concentration (Galajda *et al*., 2007), or can be used to sort mixed population of cells into different reservoirs (Mahmud *et al*., 2009). The use of nano-fabricated habitat landscapes is also very promising for the study of population dynamics of competing bacteria in shared spaces (Keymer *et al*., 2008). We are at the dawn of a new era striving to better understand and steer the behaviour of bacteria in small numbers or even at the single cell level via nanotemplates.

We can envisage further progress for instance by taming the behaviour of magnetotactic bacteria via ferromagnetic templates, designing bacterio-fobic asymmetric porous materials of practical use for medical implants, or even to exploit the recent achievements in the control of light at nanoscales (plasmonics) to guide or trap photosensitive bacteria. An interesting question that can be also tackled by nanoengineering surfaces is whether bacteria are sensitive to different degrees of wall roughness. Another fascinating concept recently posed and demonstrated by Di Leonardo and colleagues (2010) is the possibility to harvest the cooperative work of bacteria to power micromachines. Although the nanofabrication technology has reached an impressive level of development, still three-dimensional architecture will be needed to cope with the most ambitious designs of bacterial habitats.

It is worth noting that the designs used to control the motion of inert objects such as superconducting vortices, colloids or magnetic beads, are very similar to those tested in the world of small self-propelled bacteria. This direct mapping between apparently disconnected disciplines guarantees a much faster development of the research, cross fertilization of ideas and an overall huge impact far beyond the limits of microbiology. It is clear that the synergy and combined interdisciplinary effort will nourish each individual part with refreshing ideas, alternative approaches and viewpoints.

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Synthetic enzymes: catalysis on demand

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Enzymes are versatile, ubiquitous and critical to the function of almost all biological systems and can be highly selective and very efficient. Beyond their respective roles in the natural systems of origin, they have found numerous significant roles in commercial enterprises such as pharmaceuticals, foods and beverages, biofuels, detergents, biodegradable plastics, animal feed, and textiles to name a few. They can be optimized for specific industrial applications outside of their native substrate affinity, temperature, pH, etc., using a variety of genetic engineering and selection techniques. Although significant progress has been made, there remain fundamental limits to their performance that motivate the discovery of synthetic enzymes that possess all of the positive attributes of native enzymes but have superior performance in terms of lifetime and operating environments.

The notion of synthetic enzymes has been mentioned in the scientific literature as early as 1908 (Stangassinger, 1908). Since that report, researchers have been working towards synthetic enzymes that use enzymes as a reference template for 'bioinspired' artificial catalysts capable of performing identical reactions in very different chemical environments. The classic work of Pauling (1946) hypothesized that the enzyme has weak interactions with the substrate that stabilizes a transition state that induces catalysis. The design of synthetic enzymes therefore requires a deep fundamental understanding of the exact nature of this transition state as well as the sequence- and structure-based origins of enzymatic biocatalysis.

There have been several examples that highlight the potential power of synthetic enzymes. Instead of relying on proteins to serve as the catalytic scaffold, DNA has been shown as being capable of performing enzyme-like catalysis (termed DNAzymes) for the cleavage of RNA, RNA ligation, and as therapeutic agents capable of mRNA cleavage *in vivo* (Schlosser and Li, 2009). Professor Roger Sheldon's group at the Delft University of Technology has demonstrated several examples of altering the native biochemical activity of enzymes. For example, they added a metal in the active site of hydrolytic enzymes in order to mediate certain targeted chemical reactions of interest such as oxidations and through the addition of vanadium to develop a semi-synthetic vanadate phytase peroxidase (Sheldon, 2008). Other approaches have been used to create synthetic enzymes from polyleucines and other polyamino acids capable of aldol condensation between cyclohexanone and various aromatic aldehydes, as well as the enantioselective epoxidation of chalcone (Colonna *et al*., 2009). Finally, tetranuclear zinc clusters have been utilized as synthetic lipases in the enantioselective acylation of alcohols (Ohshima *et al*., 2008).

So what is there left to do in the field of synthetic enzymes that warrants excitement today? For starters, we are approaching the very real convergence of combinatorial chemistry and new fundamental understandings of enzymatic biocatalysis enabled by the current revolutions underway in the fields of high-throughput sequencing and bioinformatics. This combination gives researchers the tools required to develop new strategies of equivalent directed evolution and genetic engineering screening approaches in the discovery and optimization of synthetic enzymes capable of performing specific functions on a wide range of substrates with no loss of performance, and do so efficiently in industrial environments that would denature proteins. When combined properly with the knowledge of the specific structure- and sequence-based motifs that enable enzymes to efficiently interact with the transition state of the substrate, researchers should now be able to draw correlations from the cheminformatic and bioinformatic databases available to identify and optimize new chemomimetic synthetic enzymes. If successful, these results could have a significant impact on a diverse range of significant commercial pursuits that would benefit from the availability of synthetic enzymes, including renewable energy, artificial photosynthesis, human health, chemical and polymer manufacturing, and carbon sequestration.

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Visions about water and sanitation for the cities of the future: time to rethink environmental microbial processes

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Anno 2010, water microbiologists and process engineers are with good reason technological positivists. They are able to treat a very complex mix of chemicals and materials dissolved and suspended in water in such a way that common sewage is converted into NEWater (Qin *et al.*, 2004). It is a remarkable demonstration of technical ingenuity. Indeed, one can now eliminate major pollutants such as organics (BOD) nutrients (N and P) but also trace organics (pesticides, pharmaceuticals and personal care products) and all forms of disease-causing agents at a reasonable price of a few euro per m^3 drinking water produced, even when starting from very polluted sources such as domestic wastewaters. Yet, when looking worldwide, it appears that one out of eight persons has as yet no access to nearby drinking tap water (WHO-UNICEF, 2010). Some 1.6 million children die each year by simple diarrhoea as a result of intake of unsafe water (compare with AIDS: 280 000 victims per year). The best environmental technology that we have for the poor is SODIS, i.e. a PET plastic bottle that is exposed to sunlight for several hours (Dejung *et al.*, 2007). Indeed, only the latter technology can be applied in such regions, not imposing excessive costs for the people. From this point of view, we have to reconsider our positivism.

In the cities of the past, sanitation has always been a problem. It has gradually been resolved around 1850. Yet, the toilet with a water footprint of 40 to 70 l per capita per day will be 'unsustainable' in many places in the future. The mega cities of tomorrow have to reconsider the actual sanitation approach, which is entirely based on flushing excrements out of the city surroundings by use of ample amounts of water. Currently, 2.6 billion people have no access to decent sanitation (WHO-UNICEF, 2010). A major additional difficulty is that sanitation is taboo in many cultures, religions and let us be frank, to a large extent also in science. How much opportunity for a high level research grant does one have with a project focussed on sanitation? A remarkable exception in this respect is the Bill & Melinda Gates Foundation, providing stimuli for researchers to improve hygiene, sanitation and access to clean water (Bill & Melinda Gates Foundation, 2010).

Several microbial processes are out of date and/or out of place

The urban metabolism is at present fixed on the concept of 'disposal from the site of production' and 'total dissipation'. Food wastes, which often represent up to 10–20% of the overall energy budget of the citizen (Cuellar and Webber, 2010), must be used to be the driver of the new sanitation. Indeed, they can, together with the less appreciated faecal wastes, be digested to valuable biogas (Ma *et al.*, 2009). The anaerobic digestion process offers the possibility to recover the energy that is in the residue. Concomitantly, the overall mix is to a large extent pasteurized, particularly if the digestion is done under thermophilic conditions. Moreover, the thus hygienized residual liqour can be further used or treated to recover the fertilizer nutrients it contains. The digestion and further treatment can be done at a centralized but also at a decentralized level (Elmitwalli *et al.*, 2006; Zeeman *et al.*, 2008; Vlaeminck *et al.*, 2009). In the former case, the upgrading of the recovery product can be technically assured to a high degree. In the latter case, an incentive for the local citizens to cooperate in terms of safeguarding the overall process can be generated. In this respect, for such a drastically new approach focussed on recovery, one can roughly estimate that the costs for treating the sanitary wastes, which overall amount between 30–100 EUR per inhabitant per year, can be minimized and even in some instances might be turned to benefits (Verstraete *et al.*, 2009).

Two other aspects require consideration. First, the large amounts of water used now to flush the wastes are in many instances not available. In view of the many taboos, it appears that the NoMix toilet (Larsen and Gujer, 1996), in which urine and faeces are not mixed, offers no major obstacles to various religions and cultures (Lienert and Larsen, 2010). Hence, by designing separate lines for transport and treatment of these two types of excrements, one can considerable advance the opportunities for recovery of energy, nutrients and water. Indeed, the faecal matter can with other organic wastes (e.g. kitchen organ-

ics) be recovered in the form of a concentrated slurry, which offers much better possibilities for affordable 'Cradle to Cradle' treatment as outlined above (Verstraete *et al*., 2009). Alternatively, urine is in most cultures not considered as very offensive. Clever processes to recover the substantial amount of nitrogen and phosphorous in urine are already existing. For developing countries, a promising new nutrient recovery process is nitrification in sand filters and solar evaporation (Pronk and Kone, 2009).

Second, we should face the fact that in our current way of treating wastewater, we destroy all nitrogen by first nitrifying it and then denitrifying it to dinitrogen gas. The phosphorous, also the biologically harvested one, generally ends up in the landfill either in the form of bio-solids or as ashes from the incinerated sludge. Yet, the nitrogen represents fossil fuel (1 kg of fertilizer nitrogen requires about 1 l of fuel equivalent). Phosphorous is mainly mined in the USA, Morocco and China and the reserves are rapidly decreasing. Some forecast that they are only lasting for 50–100 years (Cordell *et al*., 2009). Hence, it stems to reason that we totally redesign our current sewage treatment because at present it is entirely conceived in the context of the past century. which had as prime message to remove and dissipate the waste, rather than to recover to the maximum the valuable components it represents.

Overall, environmental microbiologists have done a good job until now. Yet, both in the perspective of the requirements for a global sanitation and the need to generate a sustainable bio-economy, there is an urgent need to totally rethink and redesign the various currently used process lines in dealing with urban metabolism. The key process to advance is anaerobic digestion. Microbial processes to reconsider critically and most possibly to abandon in the treatment design are nitrification, denitrification, biological phosphate removal and aerobic composting. To make the cities of the future sustainable, we will have to redirect the implementation of certain microbial services. If we like it or not. And it is our duty to convince architects and urbanists that new processes and biotechnologies are ready to be implemented for new and much more clever sanitation.

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Stand alone biofuel production from algae

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Will algae keep their promise?

Microalgae have been promising for the last 60 years but so far did not prove their potential. Still many think today it will be the future feedstock for food and fuel. We believe algae will keep their promise and recently published a paper envisioning that we still need 10–15 years to develop the technology from a craft that it is today to a commercial industrial activity (Wijffels and Barbosa, 2010).

There are a few winning features of microalgae which will make this happen:

(i) microalgae are very productive;
(ii) it is the shortest route from solar energy to functional molecules;
(iii) microalgae can be grown in salt water; and
(iv) supply of nutrients like phosphate is more efficient for aqueous biomass than for land crops (phosphate will not be chemically immobilized like in soil).

The cost of production of microalgae can become a lot lower than today (Norsker *et al*., 2011) and will become cost-effective if the algal biomass is 'biorefined' (Wijffels *et al*., 2010) and there is sufficient area available for production of microalgae to provide the entire transport European fuel market with algal oil without putting any pressure on food supply (Wijffels and Barbosa, 2010).

In the USA there are many projects in the area of synthetic biology of microalgae. In comparison, much smaller budgets are available for that in Europe. On the other hand it is surprising that there is a relative lack of innovative technology development in the USA. Algal strains that were specially designed for certain purposes could never perform optimally in open systems where many process parameters cannot be controlled. In the fields of photobioreactor design, continuous operation of systems and biorefinery, which are all essential technologies for full-scale cultivation of algae, the position in Europe is stronger than in the USA. We need to integrate research on synthetic biology with innovative process principles to make progress in the field.

Apart from these optimization issues, which provide a challenging research area on which we should focus anyhow in the coming years, we would like to address three crystal balls that would turn microalgae into a very competitive crop: these are water, phosphate and CO_2. Production of microalgal oil can be done in stand alone systems if these crystal balls become reality.

Water

The fact that algae can grow on salt water makes them superior to most land crops. Because of that, algae can be grown in deserts in the condition that evaporation of water is prevented and in large ocean water bodies. For production of biofuels from land crops thousands of litres of fresh water per litre of biofuel are required while potentially in the case of microalgae, a few litres would be sufficient (Wijffels and Barbosa, 2010). That is if the technology is developed in which cooling is not done via evaporation of water but via heat exchange in the large water bodies as mentioned. Production in the open ocean would be attractive in that respect; however, we need to realize that offshore production is complex in respect to controllability and logistics and may result in an increase in production costs. If production of microalgal oils will take place in remote areas, logistics for the supply of nutrients will represent a major challenge to address. On the other hand, it provides an opportunity. An example of a nice project for nutrient recycling in offshore systems is the omega project of NASA (http://www.nasa.gov/centers/ames/research/OMEGA/index.html).

Phosphate

One of the most critical nutrients on our planet is phosphate. It is estimated that our phosphate reserves will be gone within a period of 100 years (Vaccari, 2009). Phosphates are mined, used as fertilizer for crops and taken up inefficiently because phosphate binds to the soil. As such, via production of aqueous biomass, the efficiency of phosphate utilization would increase as the phosphate supplied will not bind to soil. Nevertheless, if we produce biofuels because we run out of fossil fuels we would not solve our problem if we will run out of phosphate at similar rates instead.

Transport fuels do not contain phosphate. Ideally they consist of hydrocarbons without any phosphate. Objective should therefore be to produce algae lipids without adding phosphate. A couple of years ago we developed the concept of milking of microalgae (Hejazi and Wijffels, 2004) in which the microalga *Dunaliella salina* was used as a photocatalyst for the continuous production and *in situ* extraction of carotenoids in a two-step process. First the microalga was grown up to a certain biomass concentration after which carotenogenesis was induced and carotenoids were continuously extracted with an organic solvent. In this way carotenoids were directly produced from only CO_2, water and light energy. Our first hypothesis was that this process was conducted without damaging the cells but recently we demonstrated that direct contact between the solvent and the algae resulted in cell death of *D. salina* (Kleinegris *et al*., 2010). The concept is still very much alive, and could be potentially used if the product is excreted. As an example, cyanobacteria were developed that are able to produce and excrete higher fatty acids. New pathways were engineered for this and transporter genes were introduced (Liu *et al*., 2010). This opens the possibility to use cyanobacteria as a catalyst and to produce higher alcohols under nutrient starvation conditions in continuous culture. If such concept is further developed and applied to microalgae we will really be able to produce biofuels from algae in a sustainable way.

CO_2

An essential substrate for algae production or lipid production in microalgae is CO_2. Effective production

can only take place if the concentration of CO_2 is higher than atmospheric levels despite the greenhouse effect. Although society overproduces CO_2, it is not produced at the locations where we would like to grow algae (in deserts and on the ocean). Active supply of CO_2 to production systems at these locations would be logistically complex and very expensive. Ideally algae should grow effectively at atmospheric CO_2 pressures. A number of crops solved this problem via the enzyme carbonic anhydrase (Furbank *et al*., 1989). Carbonic anhydrase provides a CO_2 concentrating mechanism in the cell. Many microalgae also contain carbonic anhydrase and so also for microalgae this principle might be applicable (Aizawa and Miyachi, 1986). Largest bottleneck seems to be to keep the CO_2 concentration in the water phase in equilibrium with the atmospheric CO_2 concentration.

Stand alone oil production from algae

Production of algae in photobioreactors is going to be cheaper and closed systems are the only way to be able to produce microalgae without excessive needs of fresh water. If the microalgae have the ability of catalytic lipid production and excretion, nutrient inputs can be reduced substantially. Ideally algae should grow on atmospheric CO_2 such that no active supply of CO_2 needs to take place.

If (and all concepts are there) we are able to produce algae with limited amount of fresh water, without phosphate on atmospheric CO_2 we will be able to develop a stand alone system from which we only need to collect the oil. In that case all the requirements are present to develop a scalable system.

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Tapping into ancient medicine for today's problem with functional metagenomics

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Traditional Chinese medicine (TCM) incorporates an ancient set of empirical observations and multiple therapies that are at least in part congruent with recent concepts in polypharmacy (multiple therapeutic targeting) where herbal decoctions contain many biologically active natural products many of which may act synergistically (Wang *et al*., 2008). This methodology seems to be effective for managing chronic diseases, which have become a devastating epidemic worldwide. TCM is also the ultimate exemplification of personalized healthcare using non-invasive whole body diagnostic methods to stratify patients into subclasses who then receive a specifically tailored therapy often consisting of herbal decoctions, dietary and lifestyle management. Framed in this way TCM seems remarkably modern, yet we still have a poor understanding of the modes of action and relative efficacy of most TCMs compared with western drugs. Also, the laudable 'personalized and holistic nature' of TCM also makes it more difficult to apply randomized, double-blinded and placebo-controlled trials used in mainstream medicine. This has seriously hampered the integration of TCM into modern medical scheme, but with the advent of top–down systems biology tools with gut microbiota as the primary target, there may be new ways to understand and assess these therapies.

Recent evidence suggests that diet and herbal medicines interact strongly with the gut microbiome, which in turn also influences human health (Jia *et al*., 2008) and we have recently shown that population phenotype variation links to disease risk factors and that a significant part of the human metabolic phenotype may be influenced by microbial activity variation (Holmes *et al*., 2008). As most Chinese and other herbal medicines are orally administered, the host-microbial-metabolic axis (Nicholson, 2006) may be an important and as yet little studied part of TCM action and it is even possible that TCMs may work largely

by drugging or modulating this axis (Jia *et al*., 2008). Hence application of the new palette of metabolic phenotyping and metagenomic screening technologies to look at linked temporal variation of metabolites and microbes (Li *et al*., 2008) post-TCM treatment may provide new molecular evidence to verify or disprove the efficacy of such therapies. In this new functional metagenomic screening paradigm, urinary metabolites and gut bacteria would provide two non-invasive parameter windows indicative of the global health status of individual patients, thus can offer systems and quantitative assessment of health responses to TCM or any other types of alternative therapeutics.

To understand this new systems biology approach, it must be emphasized that human beings are superorganisms (Lederberg, 2000) with multiple interacting genomes including the relatively fixed and genetically inherited human karyome (*c.* 21 000 genes) and the environmentally acquired and plastic human gut microbiome (> 1 million genes). Humans and their symbionts need to be harmoniously integrated to maintain our metabolism and hence health and the functional footprints of gut metagenome can be observed in urinary polar metabolite profiles (Li *et al*., 2008).

To evaluate a TCM therapy with temporal functional metagenomics, a group of healthy people will first be selected to establish a population specific reference dataset (German *et al*., 2005), which will define the boundary of metabolic and metagenomic diversity spaces of healthy local people. Patients who receive any particular form of TCM therapy will be monitored by collecting their urine and faecal samples before, during and after the intervention together with all relevant clinical data. The metabolic or metagenomic trajectory of each patient will be calculated by comparing with all the other patients and the reference population with multivariate statistics to monitor if distance between a particular patient and the reference health population is decreased, increased or maintained in response to interventions. The reference population will define a target space towards which all the patients should move to if the intervention they receive improve their health conditions. If the intervention has some adverse effect we can expect that the patients will move in a different metabolic direction within the hyperspace and away from the reference population. In principle, this scheme can be used to test if a therapy has any effects in single individuals. This makes clinical trials for personalized therapy possible using each as their own control. This approach can also be used to evaluate efficacy or toxicity of one particular therapy to many individuals to show that if a particular measure for preventing a disease is actually working in the targeted populations. If a particular therapy showed the capacity to move the treated patients towards the metabolic space of the reference health population, the responsible biomarker variables can be identified via appropriate statistics. The identified urinary metabolites or gut bacterial species can be used as a starting point to formulate hypothesis regarding why a therapy can change these biological parameters.

This new temporal functional metagenomic approach may provide a powerful new tool for evaluating TCM therapies in the context of patient monitoring and stratification based on a holistic view of the human body including the metagenome. The integration of TCM with modern non-invasive 'omics' platforms not only offers a new lease-of-life to TCM, but could also transform this ancient empirical practice into evidence-based science and a new source of therapies for meeting 21st century global health challenges.

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Weaving a synthetic harness for the biosynthesis of natural products

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Introduction

Natural products provide a wide array of molecules with diverse biological activities, some of which have been

successfully developed into drugs for human medicine (e.g. antibiotic erythromycin, immunosuppressant rapamycin, anticancer drug doxorubicin etc). However, a number of publications in the last 5 years suggest that drug development pipelines, especially those based on natural products, are drying out in many pharmaceutical companies. Although there are several reasons for that, one of the most important one is related to frustration caused by continuous rediscovery of natural bioactive compounds in extensive screening programs. This is probably not surprising, considering the fact that many such molecules are produced by living organisms as secondary metabolites only in response to certain stimuli, which are difficult or impossible to reproduce in the lab. Consequently, an array of molecules produced in a limited set of conditions we are able to create in the lab will be overpopulated by the same secondary metabolites. At the same time, recent sequencing of genomes of several antibiotic-producing bacteria revealed that their genetic potential for production of secondary metabolites is far greater that could have been assumed based on the phenotypic data. Although now we can very often link certain genes with the biosynthesis of specific molecules (Walsh and Fischbach, 2010) important questions about the practical use of such information remain: How can we exploit such potential for discovery of new bioactive molecules? Can we make completely new 'designer' molecules with predictable pharmaceutical properties based on integration of bio- and chemoinformatics and genetic engineering? Can synthetic biology help here?

'Early birds'

Although there are yet not many examples that can support a 'yes' answer to these questions, certain trends began to emerge recently. The work by Menzella *et al*. in 2005 has demonstrated that synthetic DNA fragments encoding parts of modular polyketide synthases can be mixed-and-matched to produce functional biosynthetic enzymes (Menzella *et al*., 2005). Although not followed up, this work exemplifies the significance of rapidly evolving technology for DNA synthesis for engineering of natural product biosynthesis.

Perhaps the most important 'early bird' paper on the application of synthetic biology for the sustainable production of a drug precursor has been the one published by J.D. Keasling's group in 2006 (Ro *et al*., 2006). Clearly, this publication, describing functional 'synthetic' biochemical pathway for production of an antimalarial drug precursor, has received considerable attention from many researchers studying biosynthesis of natural products. The mix-and-match approach that supporters of 'combinatorial biosynthesis' were hoping to utilize for discovery of new drug leads received a new dimention.

So why have we not yet seen many successful examples of drug discovery-oriented projects based on synthetic biology approaches? I believe the main reason for that is reluctance of many scientists applying engineering principles to biology (synthetic biologists) to enter precarious routes of biosynthetic engineering. This is understandable, considering the biochemical complexity of secondary metabolite biosynthesis pathways, number of genes and proteins involved, several levels of regulation usually imposed by the hosts on such pathways etc. While synthetic biologists aim at creating standard connectable 'parts' and 'devices', each secondary metabolite biosynthesis pathway contains a unique component, standardization of which might represent a formidable task, or is not at all needed as a result of its uselessness in other applications. How can we engage synthetic biology into the natural product-based drug discovery programs?

Current status

Several research groups have recently reported successful heterologous expression of gene clusters for biosynthesis of natural products, although the level of molecule production remains low (reviewed in Baltz, 2010). Others have used a strategy of activating silent gene clusters to achieve production of new molecules (Gottelt *et al*., 2010). These are certainly very useful approaches expanding the chemical diversity in natural products-based drug discovery programs. However, they are still based on the traditional mode of thinking about the complex biosynthetic pathways, and are not robust enough for projects focused on drug discovery. New, out-of-the-box ideas on how complex biosynthetic pathways are functioning and how to regulate metabolic fluxes in such pathways are currently emerging. For example, the recent use of synthetic protein scaffolds in order to 'encage' anabolic enzymes and force their efficient interaction has led to a very significant increase in the efficiency of a biosynthetic pathway (Dueber *et al*., 2009). Also, a recent publication on a synthetic biology-based fine-tuning of ribosomal binding sites (RBS) aimed at optimization of protein expression (Salis *et al*., 2009) sparks new ideas. Indeed, traditionally, researchers were focusing on transcriptional regulation of biosynthetic pathways and regulation on the level of translation has been, for the most part, ignored. It seems likely that at least in some cases of failed heterologous expression of biosynthetic pathways suboptimal interaction of the host's ribosome with the heterologous RBS might be the reason for failure. If so, synthetic biology can be used to optimize RBSs within the biosynthetic gene clusters in order to adapt them to particular hosts.

Future trends

What kind of future developments can we expect in the field of natural product biosynthesis applied to drug discovery in the next 10–15 years?

Certainly, as the applications of synthetic biology principles gain momentum, and prices for *de novo* DNA synthesis continue to fall, more 'synthetic' parts will be used for re-engineering (or, using synthetic biology wording, 're-factoring') of biosynthetic pathways. Not only parts, but complete biosynthetic pathways will be synthesized *de novo* based on computer-generated design that will take into account both the genes specifying and controlling these pathways and the genetic make-ups of the hosts where these pathways will operate. For the latter, systems biology advances in understanding the metabolic networks and modelling of cell's metabolism will play a crucial role. First attempts to aid this task, and to minimize complexity of the model through minimization of host's genome have been published (e.g. Komatsu *et al.*, 2010).

I also predict that considerable progress will be made towards application of the 'combinatorial biosynthesis' principle to drug discovery using synthetic biology. Indeed, the latter opens completely new opportunities for gene combination in totally synthetic, artificial biosynthetic pathways for generation of small molecules with drug-like properties. This, however, will not be possible without significant breakthroughs in the filed of computational biology and chemistry. Ideally, as a starting point, a new pipeline for drug development would take a specific therapeutic target (e.g. an enzyme) that has to be modulated by a small molecule. Computer modelling shall generate a suggestion for a chemical structure of a small molecule that can bind the enzyme and modulate its activity. Next, a specially designed computer program based on the wealth of information available on the biosynthesis of natural products shall suggest how this molecule can be biosynthesized. Computer-based assembly of genes for biosynthetic enzymes and *in silico* fine-tuning of their expression based on the information on the host would be followed by synthesis of artificial gene cluster and its functional expression.

Wishful thinking? Yes, may be so, but the history has proven many times over that technological progress can sometimes outpace our wildest imagination. As my colleague Svein Valla has written 2 years ago in the Crystal Ball feature, 'the future is artificial', but I would add – naturally so.

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