Recombination-based DNA assembly in metabolic engineering: a goodbye to old workhorses?

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For the better part of four decades, genetic engineering has relied on a universal toolbox containing three indispensable implements: restriction enzymes, ligases and, last but not least, *Escherichia coli*. Even those of us who now languish behind laptops and in meetings rather than work at the bench instantaneously recognize the smells of Luria broth and plasmid preps.

Since the hallmark paper of Cohen and colleagues (1973), many elegant variations on the theme of restriction and ligation have been introduced to make *E. coli*-based cloning tremendously versatile. However, especially in the field of metabolic engineering, two major limitations of this trusted approach gradually became apparent. First, as the complexity of the desired DNA constructs increased, design of multi-gene constructs based on restriction and ligation turned into a molecular biologist's equivalent of Rubik's cube and the construction itself into a multi-step, painstaking and time-consuming exercise. Even 3 years ago, most of us would not have contemplated the assembly of, for example, a 20-fragment expression vector, neither as part of a PhD or postdoc project, nor in an industrial research setting. Second, the multi-step nature of DNA assembly via restriction and ligation and its requirement for unique restriction sites complicated the implementation of combinatorial approaches in the assembly of complex constructs, for example to optimize expression of a heterologous pathway by testing various combinations of enzymes from different donor organisms or by testing different combinations of promoter fragments in a multi-enzyme, heterologously expressed pathway.

During the past 4 years, fast developments in synthetic biology led to the (re)discovery and optimization of a powerful alternative approach for assembling DNA fragments that is entirely independent of restriction and ligation. This approach is based on homologous recombination of short, shared terminal sequences of the linear DNA fragments that need to be assembled and can be subdivided into *in vitro* and the *in vivo* methods.

In vitro methods for DNA assembly by recombination depend on cell-free systems, in which recombinase enzyme(s) take care of the assembly (see e.g. Gibson *et al*., 2010a). The major advantage of this method over restriction and ligation is that it enables one-step assembly of multi-fragment constructs, completely independent of the availability of unique restriction sites. The required short stretches of sequence overlap can easily be introduced, either by PCR amplification of target sequences with 'overhanging' primers or by the increasingly cost-efficient process of DNA synthesis. After *in vitro* assembly of a construct, it is transformed to a suitable microbial host, in many cases still *E. coli*.

The *in vivo* methods are based on the same principles of generation and recombination of homologous termini as the *in vitro* methods. However, they go one important step further by eliminating the need for separate assembly and transformation steps. The *in vivo* methods rely on the high efficiency of the cellular homologous recombination machinery of some microorganisms by simply transforming the required cocktail of linear DNA fragments into the host cell. When a suitable origin of replication and selection marker are included among the fragments, the transformed microorganism then faithfully recombines the fragments into an autonomously replicating episome, without any prior enzymatic treatment. *In vivo* recombination-based DNA-assembly platforms have been developed in *Bacillus subtilis* (Itaya *et al*., 2008) and in specific *E. coli rec* mutants (Datsenko and Wanner, 2000; Li and Elledge, 2005). However, these bacterial systems have a limited capacity to efficiently assemble multiple fragments and are outperformed by the incredible recombination performance of the star player of *in vivo* assembly, the yeast *Saccharomyces cerevisiae*.

In vivo assembly of DNA fragments in yeast was already proposed and demonstrated 30 years ago (Orr-Weaver *et al*., 1981; Kunes *et al*., 1987; Ma *et al*., 1987). However, it failed to really take off as a mainstay DNA construction method, perhaps mainly due to the absence of efficient techniques to generate the required homologous sequences. For a long time, its application was largely limited to the cloning of large DNA fragments that were difficult to manipulate by restriction and ligation (Larionov *et al*., 1996). *In vivo* assembly in *S. cerevisiae*, also known as transformation-associated recombination (TAR), only really caught the spotlight and took off when it was elegantly applied in the complete chemical synthesis, assembly, and cloning of a synthetic *Mycoplasma* genome by Gibson and colleagues (2010b). This milestone achievement was accompanied by a thorough evaluation of the high-fidelity assembly properties of *S. cerevisiae*, which convinced many metabolic engineers, especially those already in the yeast field, to use this new molecular biology platform for the construction of large and complex metabolic pathways (Merryman and Gibson, 2012).

As yeast biologists, we have been amazed by the power and simplicity of recombination-based assembly in our 'pet' organism when we introduced the technique into our lab. In addition to accelerating construction of unique, multi-gene constructs, the technology facilitates combinatorial approaches in metabolic engineering and, due to its

one-step simplicity, is highly compatible with automated, high-throughput strain construction. Gazing in the crystal ball, we would never dare to predict the complete demise of good old *E. coli* as a molecular biology workhorse. However, we hope and predict that, in the coming years, this simple technique for DNA assembly will find its way into many labs and especially into those that do not have a tradition in yeast molecular genetics.

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The future of sustainable fish production lies in vaccine research and development and revised regulatory measures

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A significant amount of proteins and high-quality lipids in human nutrition originates from fish and shellfish. As a result of the increasing human population and its improving health and welfare, most wild fish species have become overexploited by overfishing. The world's fish populations therefore suffer from dramatic declines. Breeding fish in analogy to breeding domestic ruminants, pigs and poultry will significantly protect endangered wild fish populations and can eventually re-establish an equilibrium of aquatic life. However, this can only be achieved by cultivating more non-carnivorous species and by changing the food requirements of carnivorous species by supplementing their nutrition with correctly processed slaughter waste materials and vegetable products. Furthermore, fish farming must not adversely affect the aquatic and terrestrial equilibrium by emissions of waste products and other pollutants.

Worldwide aquaculture has increased at an average of 9.5% per year over the past 30 years, compared with 2.6% for terrestrial meat production, freshwater production representing the highest increase. Salmon production in Norway by aquaculture, for example, has, in less than three decades, increased from virtually zero to over one million tons in 2011. In 2011 the world fisheries production of freshwater fish amounted to 11.5 megatons captured versus 44.3 megatons cultured; of marine fish 78.9 megatons captured versus 19.3 megatons cultured; and of crustaceans 6.1 megatons captured versus 5.3 megatons cultured (FAO, 2012). This leaves a vast potential for future development in aquaculture.

As with intensive animal breeding, aquaculture is strongly hampered by infectious diseases which cause massive economic losses and ecological damage. In shrimp production, a mere 10% of the juvenile shrimps leaving the hatchery reach the age of breeding maturity whereas 90% succumb mainly to infections. This leads to mostly uncontrolled use of huge amounts of antibiotics which represent a high risk to human and animal health and cause enormous environmental damage. In fish culture, antibiotics are primarily administered as medicated food whereby 70–80% of the active substances end up in the environment via food surplus and residual substances excreted from fish. Although there are no clear data on the worldwide use of antibiotics in aquaculture, estimations vary from 300 g to 1.5 kg per ton of fish or shellfish produced. This causes a significant pollution of surface waters leading to ecotoxic effects, impact on natural microbial communities and a large-scale emergence of antibiotic resistant and multi-resistant human and animal bacterial pathogens, in particular of the groups of *Vibrio*, *Aeromonas* and various *Enterobacteriaceae*. Antibiotic resistant pathogens are expected to be the main cause of unsuccessful disease therapy in human medicine in the near future, and this will probably throw back the current successful high public health status in most countries for decades. Hence a major goal that must be

