Making green

Biofuels top the list of products for many biotech companies using advanced biological engineering. Cormac Sheridan examines the diverse commercial paths being taken to reach this goal.

The $600 million research alliance on algal biofuels, which Synthetic Genomics entered into with ExxonMobil in July, represents the most persuasive evidence yet that the challenge of developing economically viable and environmentally sustainable biofuels offers a perfect proving ground for synthetic biology. The scale of the problem and the technological means of addressing it are well aligned. Effecting even a partial transition from fossil fuels to biomass-derived alternatives could substantially reduce greenhouse gas emissions in transportation—and in chemicals production, which is heavily dependent on petrochemicals—and it would spur the creation of a new industrial biotech sector.

Even though the commercial opportunity is vast, the constraints within which biofuel producers must operate are extremely tight. "The price of fuel is less than the price of bottled water, so you have to be incredibly efficient," says Jack Newman, cofounder and senior vice president of research at Amyris Biotechnologies, of Emeryville, California, one of the first movers in the field. Genetically reprogramming microorganisms on a scale that was unprecedented even five years ago holds out the prospect of achieving some of those efficiencies.

Of course, sophisticated biological manipulation alone will not deliver next-generation biofuels—the technology is only one part of a much wider effort. But many companies claim it will be difficult to contemplate any meaningful progress in the area without it.

The grand challenge

Particularly in the US, geopolitical as well as environmental considerations are behind the drive to reduce fossil fuel consumption. The US Congress, under the 2007 National Renewable Fuel Standard program, has mandated production of 36 billion gallons of all biofuels by 2022 (ref. 1). Corn-based ethanol production in the US grew rapidly during the present decade, from 1.6 billion gallons in 2000 to 9 billion gallons in 2008, according to the Washington, DC-based lobby group the Renewable Fuels Association[2]. The environmental sustainability of corn-based ethanol remains controversial, however, both from a land-use perspective and from the critical standpoint of reducing overall greenhouse gas emissions[3].

Cellulosic ethanol, produced from plant waste and other nonfood sources, offers a more attractive greenhouse gas emissions profile than its corn-derived counterpart, and the first commercial quantities of cellulosic ethanol are due to come online in 2010. One recent report indicates that some producers have either scaled back or delayed their production plans, however, because of the current economic downturn[4].

Although large volumes of cellulosic ethanol will be used in the coming decade and beyond, its long-term technical feasibility has been questioned because of its low energy density, its miscibility with water and its corrosive properties[5]. It lacks the 'drop-in-ability' of next-generation alternatives, such as butanol and alkanes, which can be handled in the existing fuel storage and distribution infrastructure[6].

Getting bacteria, yeast and algae to produce these kinds of molecules—in very large volumes, at very low cost—is the grand challenge that a slew of young biotech firms has taken on (**Table 1**). Metabolic engineering, or extensively reprogramming the physiology of the producing organisms, is the goal. Synthetic biology offers companies the means to achieve it.

"Five years ago, forget it. It would have been a dream," says Pat Gruber, CEO of Englewood, Colorado-based Gevo. His company is working on the production in yeast of isobutanol and butanol, building blocks that can be easily modified chemically to yield fuel molecules. Gruber is, however, wary of the language used to denote the techniques that Gevo and other firms are employing. "When people say 'synthetic biology,' that to me has almost no meaning. It's just genetic engineering," he says. "People are enamored with the name and the concept. It's like a [form of] branding."

Others also see synthetic biology as a continuation of classical genetic engineering, albeit with a greater degree of intensity. "When I think of synthetic biology, I think of all the things I used to do as a biologist, slowly, methodically, and not always with a lot of success," says Stephen del Cardayre, vice president of research and development at LS9 of South San Francisco, California. "Today, most of these methods can be automated such that robots can carry out thousands of experiments effectively, efficiently and with excellent success. This allows us to test many, many more hypotheses in parallel—quickly and cost effectively."

Improving on nature

But the present effort goes beyond a massive industrial scale-up of genetic engineering. The ready availability of custom-designed synthetic DNA molecules marks a significant point of departure from earlier eras of genetic manipulation. Amyris Biotechnologies, for example, is engineering yeast cells to make a class of branched hydrocarbons called isoprenoids using biosynthetic genes that are ultimately derived from plants, but it rarely works with actual plant DNA. "You might generate a lead that way, but by the time that piece of DNA sees a microbe it's been through DNA synthesis," says Newman. And more often than not, the immediate 'source' of a gene is a database rather than a living plant.

Using synthetic DNA is not simply a matter of speed or convenience. The redundancy of the genetic code enables scientists to improve the expression of foreign genes in different hosts by designing synthetic DNA molecules that encode the correct protein but have a nucleotide sequence that takes account of the host organism's codon usage bias. As Newman observes, DNA contains a lot more information than just an amino acid sequence. "There's a whole rule set about translatability we haven't even formulated." Design procedures that are 'translation aware' are beginning to emerge, however.

Verdezyne (formerly Coda Genomics), of Carlsbad, California, has developed a computational biology platform for designing self-assembling synthetic genes that not only addresses codon usage bias but also takes into account a lesser-known phenomenon, codon pair bias[7]. The over-representation of certain codon pairs in an RNA sequence appears to act as a brake on translation. Altering them can boost gene expression. Including more of them in a sequence can slow it down. "It's not just the codon abundance but the codon context that affects translation," says Verdezyne CSO Stephen Picataggio.

Jonathan Wolfson, cofounder of the company Solazyme, shows green algae that his company is engineering to create renewable biofuels.

Table 1 Selected advanced biofuel companies

Company	Process	Producing Organism
Ethanol		
Algenol Biofuels, Naples, Florida	Photosynthesis in contained bioreactor	Cyanobacteria
BioGasol, Ballerup, Denmark	Glucose & xylose fermentation	Yeast, anaerobic, thermophilic bacteria
Codexis[1], Redwood City, California	High performance enzymes via gene shuffling	Not available
Coskata, Warrenville, Illinois	High temperature biomass gasification and fermentation using carbon monoxide and hydrogen	Not disclosed
Dupont Danisco Cellulosic Ethanol, Itaca, New York	Combined cellulosic conversion and fermentation	*Zymomonas mobilis*
Gevo, Englewood, Colorado	Production of higher alcohols via amino acid biosynthetic pathway	Yeast
Green Biologics, Abingdon, UK	Modified classical acetone, butanol ethanol (ABE) fermentation	Clostridium species, Geobacillus species
Joule Biotechnologies, Cambridge, Massachusetts	Helioculture modified photosynthetic process in closed bioreactor	Modified photosynthetic organisms
Lanza Tech, Auckland, New Zealand	Fermentation process using carbon monoxide and hydrogen from syngas and fluegas	Not disclosed
LS9, S San Francisco	Biodiesel fermentation via fatty acid metabolism	*E. coli*
Mascoma, Lebanon, New Hampshire	Combined lignocelulose conversion with fermentation	Yeast, *Clostridium thermocellum*
Qteros, Marlborough, Massachusetts	One-step bacterial lignocellulose conversion and fermentation	*Clostridim phytofermentans*
TMO Renewables, Guildford, UK	Combined cellulosic conversion and fermentation	Geobacillus TM242
Verdezyne, Carlsbad, California	Improved yeast fermentation based on microbial glycolytic pathway and xylose isomerase	Yeast
Verenium, Cambridge, Mass	Combined cellulosic conversion and fermentation	Ethanologenic bacteria
Zeachem, Lakewood, Colorado	Hybrid biochemical and thermochemical process involving acetic acid fermentation	Naturally occurring acetate producing bacteria
Diesel		
Amyris Biotechnologies, Emeryville, California	Isoprenoid biosynthesis via mevalonate pathway	Yeast
Aurora Biofuels, Alameda, California	Photosynthesis in open pond system	Naturally occurring algae
OPX Biotechnologies, Boulder, Colorado	Undisclosed	*E. coli*
Algal oils		
Sapphire Energy, San Diego, California	Photosynthesis	Photosynthetic algae
Solazyme, S. San Francisco	Photosynthesis	Photosynthetic algae
Solix Biofuels, Fort Collins, Colorado	Photosynthesis in close system photobioreactor	Photosynthetic algae
Synthetic Genomics, La Jolla, California	Combined phtosynthetic production and secretion	Photosynthetic algae

[1]Codexis is a technology provider to cellulosic ethanol producer logen, of Ottawa, Canada.

His company has successfully applied this approach to the expression of the bacterial enzyme xylose isomerase in yeast, enabling it to ferment the five-carbon sugar, which is a significant cellulose constituent. "People have been trying to do this for 35 years already. I tried it as a postdoc," Picataggio says. "The problem has been the enzyme misfolds in the yeast cytoplasm."

DNA 2.0, of Menlo Park, California, is also working on the role of codon usage in synthetic gene expression, and it recently published data suggesting that codons used to encode a subset of amino acids were strongly correlated with expression of two genes in *Escherichia coli*[8].

Nature, however, remains the starting point for any gene (or protein) engineering effort, even though the final molecule may undergo many alterations. "We don't think that we understand enzymes nearly well enough to sit down and design the perfect one," says Lori Giver, vice president of systems biology at Codexis, of Redwood City, California, which uses DNA shuffling to evolve high-performance enzymes.

The fork in the road

Although all the firms working on advanced biofuels share similar goals, the specifics of their technologies and their business strategies differ. Given the field's early stage of development—and the commercial rewards at stake—some firms are reluctant at this stage to divulge fully their technology strategies. But each company has a fundamental decision to make: whether to engineer a biofuel-producing capability into a well-known, robust industrial organism or to engineer industrial fitness and other necessary attributes into an organism that is a natural producer of the molecule of interest.

Extensive genetic tools and components are available for engineering yeast and *E. coli*. These also have long histories as fermentation organisms. The same cannot be said for photosynthetic algae or bacteria, although such organisms are attractive because they require no raw materials, other than sunlight, carbon dioxide and water, to make hydrocarbons. Joule Biotechnologies, of Cambridge, Massachusetts, is one firm taking the photosynthetic route to biofuels. Photosynthesis offers the overarching advantage of bypassing the need to break down the lignin and cellulose present in plant biomass. "The notion of really doing it directly is where you get quite a lot of your efficiency," says company cofounder David Berry, of Cambridge-based Flagship Ventures (which has also funded LS9 and Lebanon, New Hampshire-based Mascoma). Joule has developed proprietary methods for manipulating its target organisms. "The way I like to describe it is recapitulating about 30 years of *E. coli* engineering in about eighteen months," says Berry. The company has not identified the organisms it is working on, however. "They are naturally photosynthetic organisms we've engineered in a number of new ways," Berry says.

Synthetic Genomics, of La Jolla, California, and ExxonMobil, of Irving, Texas, are also keeping specific details of their research program

under wraps for now, although the scale of the alliance suggests that it will have a very broad scope. "We're going to be testing probably every approach that's out there," says Synthetic Genomics CEO and cofounder J. Craig Venter. Synthetic Genomics scientists have engineered algal strains that can transport lipids out of the cell, which offers the possibility of setting up a continuous biomanufacturing process rather than an intermittent cycle of growing and harvesting. "The conventional wisdom did not have algae that secreted hydrocarbons in a pure form into the media—so I think our breakthrough on that front changes the entire equation," Venter said last July. Although its approach has yet to be scaled up, the early indications are promising. "The existing starting yields that we have are on the order of ten times more efficient than acreage for production of corn and we hope to substantially build on that through this program," Venter went on.

Around a month after the ExxonMobil announcement, Venter and colleagues at the J. Craig Venter Institute (JCVI), of Rockville, Maryland, reported that they had succeeded in transferring an entire bacterial chromosome—that of *Mycoplasma mycoides* ssp. *mycoides*—into a yeast cell and then subjecting it to modification using the yeast genetic system[9]. If applicable to other organisms, such as biofuel producers, it could provide a general method for engineering the genomes of organisms that are otherwise difficult to manipulate. "It will be a key enabling technology for the whole field," Venter says. "It creates the ability to do rapid changes that were not remotely possible before."

The road map to success

The traits that biofuel companies want to engineer into producing organisms extend far beyond those directly associated with the production of a specific fuel molecule. The specific changes that individual companies make—and the methods they use to make them—obviously vary. Michael Lynch, founder and CSO of OPX Biotechnologies, of Boulder, Colorado, likens the problem to driving from New York to Los Angeles without a road map. Companies that build up a large-scale, automated genetic engineering platform are, he says, building a faster car or plane. "We focus a lot on how you map out the space between here and there." OPX takes a "population-based approach" to mapping the links between genotypes and phenotypes. "We perturb the network and measure the results in a massively parallel way," Lynch says.

All strain improvement efforts have to operate within the boundaries imposed by microbial physiology. "Each time you do some permutation it impacts the energy balance," Gevo's Gruber says. If a biosynthetic pathway is to be massively overexpressed, it requires a steady supply of the appropriate cofactors, for example. Pathways that consume energy may need to be switched off. "You have to prune the metabolic pathways you don't want," says Jim Flatt, president of Mascoma. Mascoma is developing what it terms consolidated bioprocessing technology, which aims to combine the hydrolysis of lignocellulose and the fermentation of the resulting sugar molecules to ethanol in a single process. It involves engineering cells—bacterial or yeast—to express and secrete a suite of cellulase enzymes to break down cellulose and hemicellulose into their constituent sugars and to convert those sugars to ethanol. Hydrolysis of cellulose and hemicellulose requires about 20 distinct enzymes—that are normally provided by commercial suppliers such as Novozymes, of Bagsvaerd, Denmark, or the Genencor unit of Copenhagen, Denmark-based Danisco. "It's basically a chamber orchestra of activity you need here—it's not just breaking down starch, which is an easy process," says Flatt. The hydrolysis process can also result in the production of byproducts, including acids, ketones and aldehydes, that can inhibit the growth of cells as well as the secreted enzymes. Mascoma is working on directed evolution strategies to adapt cells so that they not only tolerate but thrive on these molecules, Flatt says.

LS9's del Cardayre emphasizes the advantages of harnessing biosynthetic pathways involved in the production of primary metabolites, as a large fraction of the cell's normal metabolic flux will be directed toward that pathway in any case. His firm is focused on the production of alkanes from fatty acid intermediates in *E. coli*. "Just before the fatty acid intermediates are incorporated into the cell membrane, we steal them from that pathway and divert them into a fuel biosynthetic pathway we've engineered into the cell," he says. Gevo is also focused on a primary metabolic pathway. It is building on technology in-licensed from James Liao of the University of California, Los Angeles, who demonstrated how to divert intermediates from *E. coli*'s amino acid biosynthetic pathway toward the production of branched chain alcohols[10].

Industrial scale biofuels

Scaling up any of these processes represents a daunting challenge. What works on a lab bench will not necessarily work in an industrial fermentation vessel. Gevo, which is pursuing a retrofit strategy based on migrating corn ethanol plants over to butanol production, recently opened its first demonstration-scale facility. Amyris opened a demonstration-scale production facility in Brazil midyear and aims to produce commercial quantities of its renewable diesel from sugarcane feedstock in 2011. LS9 says it will reach demonstration-scale production of its UltraClean Diesel fuel in 2010. The availability of cost-effective enzymes for breaking down cellulose will be critical for the success of the field, del Cardayre says. "We are rooting for and working with those companies developing technologies for converting biomass cost-effectively into sugar."

Although it is evident that synthetic biology is central to the development of advanced biofuels, it is not yet clear whether a fully synthetic genome will ever be deployed in a live production environment. "A fully synthetic microorganism may not have the robustness which is needed for large-scale industrial bioprocesses," Picataggio says. Venter says the first generation of producing organisms he is working on could potentially be a naturally occurring strain. Further generations will be what he calls "synthetic genomic constructs," with partially synthetic genomes.

In any case, economics, not technology, will be the ultimate arbiter of success. The list of successful industrial fermentations "is not long," Gruber notes. The most noteworthy include the production of commodities such as ethanol, lysine, citric acid, lactic acid, polyhydroxyalkanoate, 1,3-propanediol and erythritol. To be competitive, he says, a fermentation needs to produce around 100 grams per liter of end product; its productivity should exceed two grams per liter per hour; and its anaerobic yield should stand at ~95% of the theoretical yield. "If you meet those requirements, you will be in economically efficient space," he says.

The synthetic biology pioneers will have to clear these hurdles just as their industrial microbiology predecessors did previously. Although buzzwords and dazzling science have hyped expectations, considerable challenges lie ahead before new genome engineering applications can be turned into green gold. As Gruber puts it: "I've never seen anything commercially successful in our industrial biotech space that started with, 'Gee whiz, this is a cool invention.'"

Cormac Sheridan, Dublin

1. US Environmental Protection Agency. EPA proposes new regulations for the renewable fuel standard program for 2010 and beyond. (EPA-420-F-09-023) <http://www.epa.gov/otaq/renewablefuels/420f09023.htm#3> (2009).
2. Renewable Fuels Association. Ethanol industry statistics. <http://www.ethanolrfa.org/industry/statistics/#A> (2005–2009).
3. Searchinger, T. *et al. Science* **319**, 1238–1240 (2008).
4. Kwok, R. *Nature* **461**, 582–583 (2009).
5. Keasling, J.D. & Chou, H. *Nat. Biotechnol.* **26**, 298–299 (2008).
6. Lee, S.K. *et al. Curr. Opin. Biotechnol.* **19**, 556–563 (2008).
7. Larsen, L.S.Z. *et al. Int. J. Bioinform. Res. Appl.* **4**, 324–336 (2008).
8. Welch, M. *et al. PLoS One* **4**, e7002 (2009).
9. Lartigue, C. *et al. Science* **325**, 1693–1696 (2009).
10. Atsumi, S. *et al. Nature* **451**, 86–89 (2008).