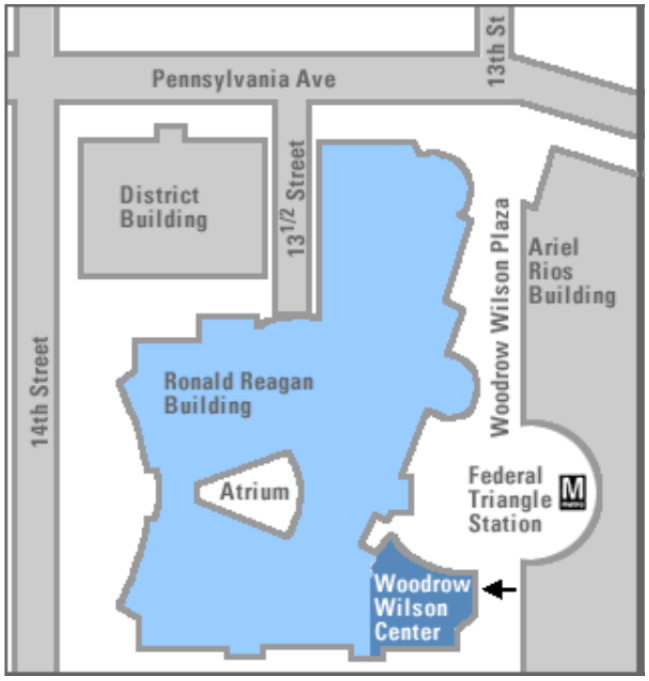
Comprehensive Environmental Assessment and Synthetic Biology Applications Workshop
The Woodrow Wilson International Center for Scholars, Science, Technology & Innovation Program
Ronald Reagan Building and International Trade Center, 1300 Pennsylvania Ave., NW, Washington, DC
Thursday, July 28th, 2011, 8:30am to 4:30pm



ESSENTIALS
Location: 1300 Pennsylvania Ave, NW, in the Patrick Moynihan Boardroom on the **6th floor**. Due to security regulations, please allow extra time to enter the building. A photo I.D. is **required** for entry. The Federal Triangle Metro Station is located at the Ronald Reagan Building.

Materials: Notebooks with hardcopies of these emailed materials and updated information will be provided at the Wilson Center on the day of the workshop.

Meals: Light breakfast (8:30-9:00 AM), coffee breaks and lunch will accommodate vegetarians, carnivores and omnivores. We will hold an optional dinner on Thursday evening for interested participants. Please contact Genya Dana to indicate if you will be staying for dinner, if you have not already done so.

Further Information: Please contact Genya Dana at 612-327-4900 (cell) or 202-691-4320 (work) or Genya.Dana@wilsoncenter.org; or Todd Kuiken at 202-271-6777 (cell) or 202-691-4398 (work) or Todd.Kuiken@wilsoncenter.org.

WORKSHOP AGENDA (draft)

8:30 Breakfast

9:00 Opening remarks
Welcome and introduction—*Dave Rejeski , Director, WWC Science Technology & Innovation Program*
Purpose and structure of workshop—*Genya Dana, WWC Science Technology & Innovation Program*
Participant introduction

9:15 Introductory presentations
S. elongatus and sucrose production—*Daniel C. Ducat & Patrick Boyle, Harvard*
Important gene flow concepts—*Allison Snow, Ohio State (via phone conference)*
CEA framework and approach—*Genya Dana*
Case study scenario of *S. elongatus* in a photobioreactor system—*Genya Dana*

10:25 Begin workshop exercises
Topic 1: Lifecycle of *S. elongatus*
Q1: What may be potential escape routes from the production system?
Q2: What may happen to the organism upon escape: e.g., does it survive?
Q3: What might be important research questions re: escape routes and organism survivability?

11:15 Break

11:30 Topic 2: Environmental compartments
Q4: Which environmental compartments may be exposed?
Q5: What external factors might influence escape and movement into the compartments?
Q6: What kinds of research questions would be important to answering Q4 & 5?

12:30 Lunch

1:15 Topic 3: Organisms
Q7: Categories of organisms—which one(s) may be particularly important to consider?
Q8: What external factors might influence exposure/uptake of the organism or DNA?
Q9: What kinds of research questions would be important to answering Q7&8?

1:45 Topic 3: Ecological processes
Q10: Categories of ecosystem processes—which one(s) may be particularly important to consider?
Q11: What external factors might influence ability of *S. elongatus* to influence ecosystem processes?
Q12: What kinds of research questions would be important to answering Q10 &11?

2:15 Break

2:30 Topic 4: Effects
Q13: What kind of effects might be important to investigate and at what organizational level?
Q14: What external factors might influence the potential effects of *S. elongatus*?
Q15: What kinds of research questions would be important to answering Q10 &11?

3:00 Consolidate preliminary research priorities
Each participant individually puts forward their top research priority and makes a case for it

3:30 Feedback on information generated

Example questions:

- Did certain pathways emerge as important?
- Did we identify any issues that can be resolved in the near term through risk management actions?
- Are the information gaps that we generated here generalizable? For example, would you want to know the same things regardless of the syn bio application or production system?

4:00 Feedback on CEA framework

Example questions:

- Is this framework helpful for organizing information and identifying research gaps and risk management opportunities?
- Is it missing key aspects or concepts?
- Is this a useful tool for engaging stakeholders?

4:20 Next steps
Linkages with Wilson Center and SynBERC synthetic biology and public policy projects—*Ken Oye, MIT; Dave Rejeski, WWC STIP; Genya Dana, WWC STIP*

4:30pm Workshop adjourns

5:00 Dinner for interested participants

DESCRIPTION AND PURPOSE OF WORKSHOP

In this workshop, an interdisciplinary group of engineers and ecologists will investigate the utility of a Comprehensive Environmental Assessment (CEA) framework to help identify important research questions useful for supporting future ecological risk assessments of synthetic biology applications. The US Environmental Protection Agency has been using the CEA approach to identify risk-related research priorities for nanomaterials; it may also be applicable to synthetic biology applications at early stages of research and development. The CEA approach guides the comprehensive consideration of how novel organisms or materials, at each stage of their lifecycle, may move through and impact the environment; it combines a lifecycle analysis approach with a risk assessment framework. Participants will use a case study of a cyanobacteria engineered to produce sucrose (developed by researchers at Harvard University) to 1) develop a list of priority ecological impacts-related research questions, 2) identify potential early stage risk management actions, and 3) develop a better-informed, interdisciplinary community of scientists and stakeholders. Workshop discussion will be organized around the CEA framework. There will also be opportunities for all participants to reflect on emerging themes and the applicability of CEA to other synthetic biology applications.

Please refer to the following documents for more information about the full extent of the US Environmental Protection Agency's Comprehensive Environmental Assessment approach:

U.S. EPA (2010a) Nanomaterial case studies workshop: Developing a comprehensive environmental assessment research strategy for nanoscale titanium dioxide. EPA report 600/R-10/042 (http://www.epa.gov/osp/bosc/pdf/nano1005summ.pdf)

U.S. EPA (2010b) Nanomaterial case studies: Nanoscale titanium dioxide in water treatment and topical sunscreen (final). EPA report 600/R-09/057F (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=230972)

U.S. EPA (2010c) Nanomaterial case study: Nanoscale silver in disinfectant spray (external review draft). EPA report 600/R-10/081 (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=226723)

BACKGROUND ON COMPREHENSIVE ENVIRONMENTAL ASSESSMENT (CEA) APPROACH

The Woodrow Wilson Center's Synthetic Biology Project is piloting a Comprehensive Environmental Assessment (CEA) framework for its ability to guide the identification of important research questions to support future ecological risk assessments of synthetic biology applications. The first pilot exercise, on July 28, 2011, will focus on *Synechococcus elongatus*, a cyanobacteria engineered to produce sucrose in a photobioreactor system. The CEA framework guides the holistic evaluation of how novel organisms or materials may, at each stage of their lifecycle, move through and impact the environment (Figure 1); it combines a lifecycle analysis approach with a risk assessment framework. The CEA approach is one tool used by the US Environmental Protection Agency to evaluate nanomaterial risks and identify research directions[1]; it may also be applicable to synthetic biology applications at early stages of research and development.

The CEA pilot is part of a larger Wilson Center endeavor to prototype risk assessment methods from other fields (e.g., invasion biology, engineering) for their utility in identifying early-stage hazards and research directions for synthetic biology applications. The Center's Science, Technology and Innovation Program has implemented similar projects with emerging technologies. For example, they recently facilitated development of a comprehensive nanotechnology risk framework through an Environmental Defense—Dupont Partnership.[2]

Purpose of the CEA: The CEA framework encourages users to think holistically about the steps necessary for an organism or material to enter the environment (e.g., lab escape, purposeful release), what it might come in contact with (e.g., animals, plants, microorganisms), what potential impacts might be (e.g., gene flow, disruption of ecological processes), and what factors influence these actions (e.g., pH, species behavior, biosafety protocols). The process of working through the CEA encourages interdisciplinary thinking and collaboration between stakeholders, with the goal of identifying important research areas to support future risk assessments.

Outputs of the CEA: In the context of identifying important risk-related research questions for synthetic biology, the CEA approach can help identify:

1) key lifecycle stages where exposure may be likely,
2) key pathways of environmental exposure,
3) important external factors influencing the movement and fate of the organism,
4) priority areas of research useful for supporting future risk assessments, and
5) areas for improvement of existing (or designing new) biosafety measures.

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[1] U.S. EPA (2010a) Nanomaterial case studies workshop: Developing a comprehensive environmental assessment research strategy for nanoscale titanium dioxide. EPA report 600/R-10/042 (http://www.epa.gov/osp/bosc/pdf/nano1005summ.pdf)
U.S. EPA (2010b) Nanomaterial case studies: Nanoscale titanium dioxide in water treatment and topical sunscreen (final). EPA report 600/R-09/057F (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=230972)
U.S. EPA (2010c) Nanomaterial case study: Nanoscale silver in disinfectant spray (external review draft).
EPA report 600/R-10/081 (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=226723)

[2] (http://nanoriskframework.com/page.cfm?tagID=1095).

Comprehensive Environmental Assessment (CEA) Framework

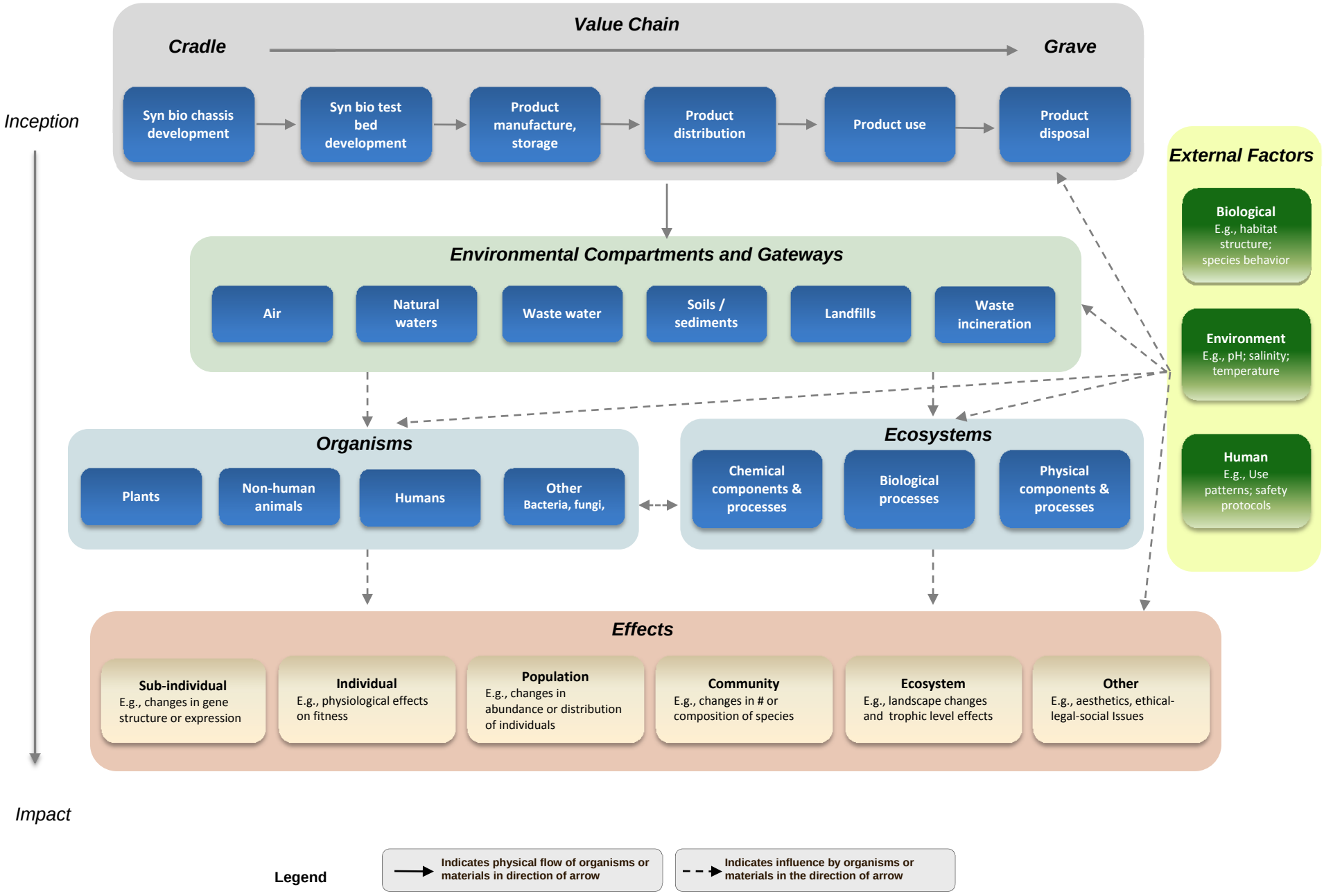

Figure 1. Comprehensive Environmental Assessment (CEA) Framework (modified from U.S. EPA (2010c) Nanomaterial case study: Nanoscale silver in disinfectant spray (external review draft). EPA report 600/R-10/081 (http://cfpub.epa.gov/ncea/cfm/recordisplay.cfm?deid=226723)

BACKGROUND MATERIAL ON cscB-EXPRESSING *SYNECHOCOCCUS ELONGATUS*

Production of Sugars using Cyanobacteria: Technical Overview

Produced by Daniel C. Ducat and Patrick Boyle
Systems Biology Department, Harvard Medical School, Boston, MA and
Wyss Institute for Biologically Inspired Engineering, Boston, MA

Background:

Today's biotechnological methods for the production of fuels, chemicals, and therapeutics rely heavily upon carbohydrate inputs. The sugars used as feedstocks for such biologically-driven production are almost exclusively derived from agricultural plants and/or cellulosic breakdown products. Although biologicals are being touted as sustainable alternatives to chemicals and fuels produced from petroleum because they can be carbon-neutral and renewable, additional concerns are raised by the processing of plant matter into fuels and chemicals. The most common argument is that plant crops destined for bioindustrial use generate competition with standard, edible crops for arable land and/or directly compete with food markets, raising food prices and insecurity. Additional concerns typically relate to the (in)efficiencies in the conversion of some popular, yet suboptimal carbohydrate to chemicals, such as in the corn to ethanol program of the US.

Herein we detail the efficient production and export of a sugar feedstock, sucrose, from the freshwater cyanobacterial species *Synechococcus elongatus PCC 7942 (S. elongatus)*. Cyanobacteria represent a potentially interesting alternate source for carbohydrates from plants because they are simple, single-celled organisms that have an exclusively aquatic life-cycle. As such, cyanobacteria could be grown in areas of with poor soils, or in coastal regions – greatly reducing land competition with other commercial crops or natural areas of high primary productivity and biodiversity. Furthermore, cyanobacteria are naturally more efficient at converting solar energy to biomass than standard crops (~3-5% solar conversion, compared to 0.1-0.3% conversion by most land plants). Finally, species of cyanobacteria such as *S. elongatus* grow autotrophically in water (needing only sunlight and carbon dioxide) with very little other requirements, potentially minimizing the need for fertilizers and associated run-off.

The Silver Lab at Harvard Medical School is currently investigating strains of cyanobacteria that are capable of producing and exporting sucrose in a highly efficient manner. If levels of production can be scaled effectively, we anticipate that cyanobacteria of this nature could produce simple sugars at a per hectare rate that greatly outstrips current levels generated by corn and which may even exceed those of more-effective programs of sugarcane.

Sucrose-producing cyanobacteria:

The underlying principal of our cyanobacterial production method for sugars relies on the fact that sucrose is accumulated as a "compatible solute" in some cyanobacteria under certain environmental conditions. A compatible solute is essentially a biological molecule which appears to be highly compatible with the interior chemistry of a cell. As such, compatible solutes are inherently able to be concentrated in cells at levels that would be toxic for most other metabolites; sucrose has been

observed in cells at concentrations nearing 1M under some conditions. Compatible solutes like sucrose are thought to have additional, poorly understood properties to protect cells from environmental stresses, such as desiccation, high or low temperatures, and osmotic stresses.

When *S. elongatus* is exposed to salty environments, the difference in the dissolved ions outside the cell creates an osmotic pressure that tends to pull water from the cell, causing desiccation. Freshwater species of cyanobacteria often adapt to these environments by accumulating a counter-ion to balance the salts, thereby preventing the loss of water; *S. elongatus* upregulates the production of sucrose (up to nearly 0.5M) for this purpose. Conversely, without environmental pressures, sucrose is not a major biochemical component of *S. elongatus* cells, and internal carbohydrates are usually in the form of other simple sugars, or stored as chains of glucose (glycogen).

To engineer a strain of cyanobacteria that would export internal sucrose, we introduced a sucrose transporter gene from the common lab bacterium, *Escherichia coli* (*E. coli*). This gene is also found in many wild-type *E. coli* strains. This gene encodes the sucrose permease, cscB, which actively transports sucrose across the cell membrane by taking advantage of proton (H+) gradients (Figure 1). In *E. coli*, cscB functions to import sucrose from the environment, taking advantage of the fact that this bacterium typically grows in environments that are more acidic than the interior of the cell. In contrast, *S. elongatus* naturally alters the environment it grows in to make it more basic. Thus the same gene will act in reverse when engineered into these cyanobacteria, driving sucrose out of the cell.

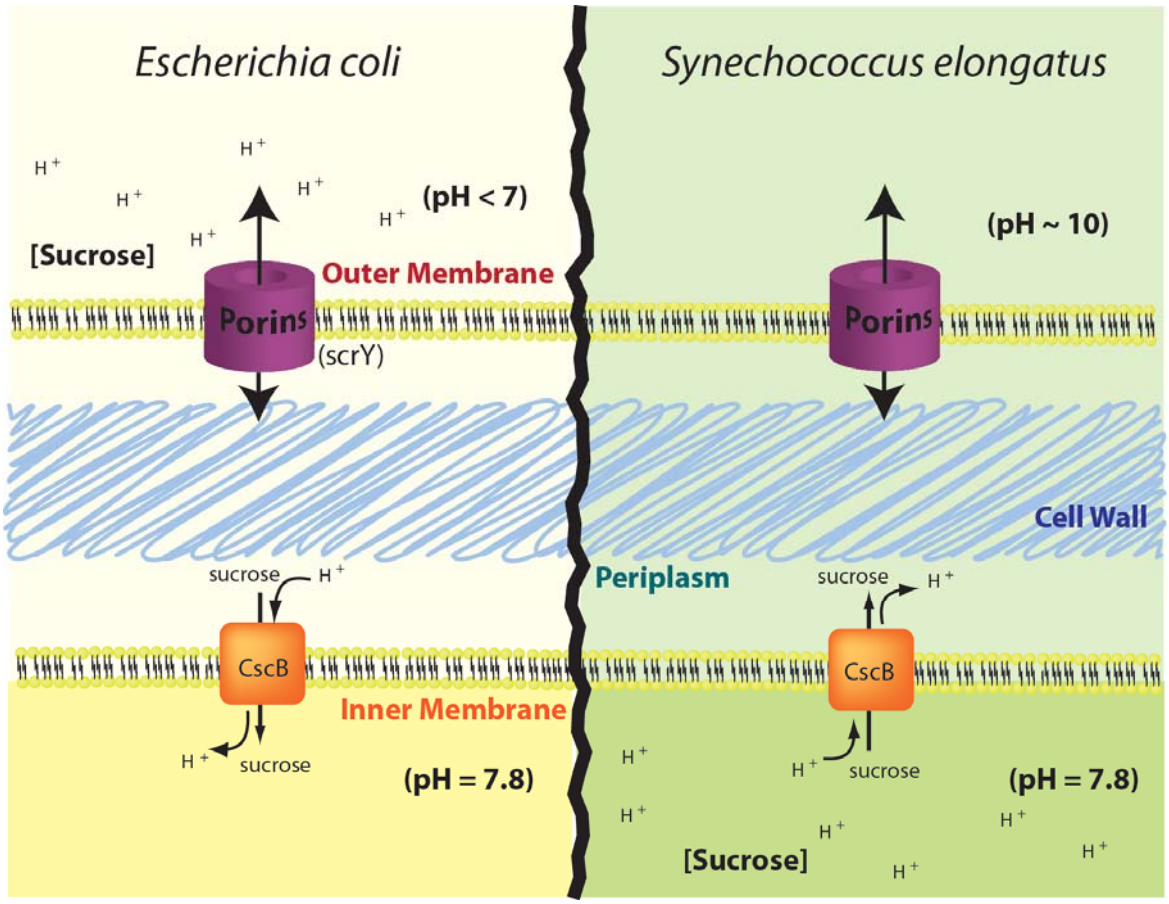

Figure 1. Sucrose transport in *E. coli* and *S. elongatus*. Sucrose permease (cscB) acts to transport sucrose across the inner membrane into the cytoplasm of *E. coli*, allowing this bacterium to use this sugar as an energy source. While the acidic environment of *E. coli* is coupled to the import of sucrose, *S. elongatus* tends to grow in basic environments – so the direction of transport is reversed, leading to the export of internal sucrose. The outer membrane of most cells, including *S. elongatus*, contains open pores that can non-specifically transport a variety of small molecules, including sucrose, allowing for the complete export of such substances.

We generated a genetic construct where the cscB gene was placed under the control of a genetic promoter that is responsive to an external commonly-used chemical inducer (IPTG). In this design, the sucrose permease gene is usually off, and is only activated when IPTG is added to the culture media. To introduce the construct into *S. elongatus*, we flanked this sequence with DNA identical to that of a particular location in the natural *S. elongatus* genome. We then integrated this construct into the genome of *S. elongatus* using the natural tendency of these cyanobacteria to take up DNA from the surrounding environment. Once in the cell, the flanking DNA directs the construct to be integrated within the *S. elongatus* genome at that particular location by a process that mimics the natural DNA repair mechanisms of the cell. We differentiated cells containing the cscB gene by co-integration of an antibiotic resistance gene; however this antibiotic resistance would be removed before any large-scale applications.

The final strain of *S. elongatus* has an inactive cscB gene until IPTG is introduced into the media, whereupon the gene is transcribed into mRNA and translated into protein. The cscB protein is inserted into the inner membrane by the cellular machinery, and will bind to and export any sucrose in the cytoplasm (Figure 1). When salt is added to the water containing *S. elongatus*, the cyanobacteria produce internal sucrose to balance the osmotic stress and protect the cell. Therefore, our engineered pathway is only active in the presence of both salt (to induce the formation of sucrose) and IPTG. We anticipate that strains of cyanobacteria designed for eventual use in a large-scale plant would have the cscB gene regulated in a manner independent of IPTG to reduce the cost of activating sucrose production. For example, the gene could be designed to be constantly on (constitutive), or under the regulation of a more inexpensive inducer.

Figure 2 shows the capacity of *S. elongatus* to produce sucrose when in the presence of both salt and IPTG (Figure 2; cscB induced).

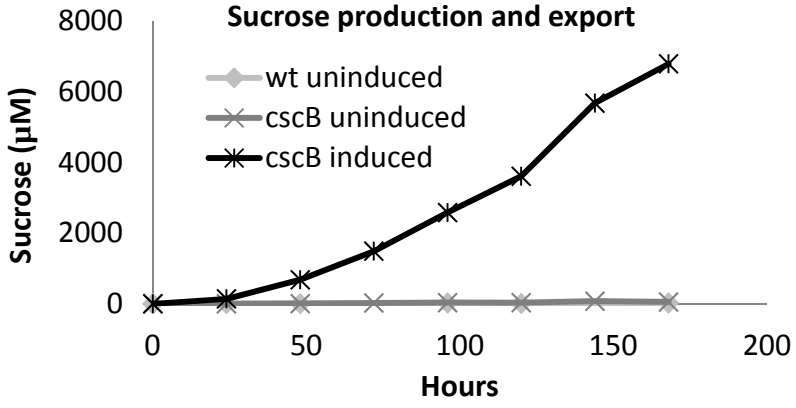

Figure 2. Production and export of sucrose in *S. elongatus.* Production and export of sucrose in *S. elongatus* over 1 week through the regulated expression of sucrose permease. Sucrose export in wild-type and uninduced cells remains nearly undetectable.

In salty waters (100-200mM NaCl), *S. elongatus* expressing cscB actively export sucrose into culture media at rates up to ~30 mg L^{-1} hour illumination^{-1}. Since sucrose is not reabsorbed during periods of darkness, sucrose accumulates to concentrations >10mM without toxicity or the requirement for complex removal schemes. Furthermore, when dilute cultures of cyanobacteria are allowed to grow while producing sucrose over time, the rates of sucrose production increase as the density of the culture increases without the need to remove external sucrose (Figure 3). This is an important distinction because most biologically produced molecules eventually cause toxic effects when generated at high concentrations. This production method can allow for the generation and accumulation of sucrose without the need for complicated schemes to remove the sucrose prior to harvesting.

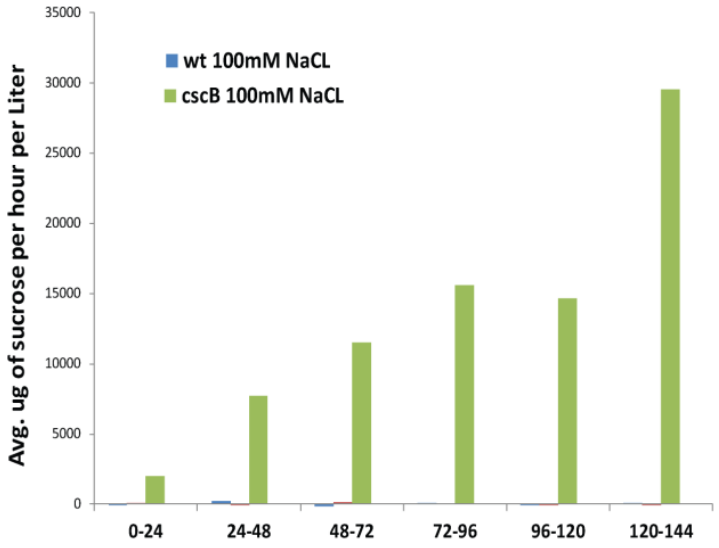

Figure 3: Sucrose production over time. Sucrose production by a dilute culture of *S. elongatus* increases across the period of a week as a function of increased growth of the cyanobacterial culture. High levels of production could be maintained without toxic effects in concentrated samples.

The rate of sucrose production displayed above is such that sugars could be generated from cyanobacteria at higher rates than existing land crops. While the data displayed are obtained under laboratory culture conditions that may not be realistic in large scale, even conservative estimates from these data would suggest a much more efficient production than achieved by corn-derived sugars. More optimistic estimates of production of 0.5mM sucrose per day with 12 hours of sunlight (we regularly achieve 1mM+ under laboratory conditions) would result in ~8-14 tons of sucrose per hectare per year, depending on the estimated liters per hectare of a cyanobacterial 'farm' and the number of days in the growing season. If achieved, this would represent a source of sucrose competitive with that of sugarcane grown in tropical climates, such as Hawaii or Brazil.

Information relevant to cscB-expressing *S. elongatus* and associated sucrose production:

- *S. elongatus* naturally requires minimal nutrients for growth. The most notable component of the media we use to culture *S. elongatus* is the presence of a nitrogen source ($NaNO_3$). We also add low concentrations of sulfate ($MgSO_4$), potassium (K_2HPO_4), and small quantities of water softening agents (Na_2CO_3 and EDTA). Finally, we add trace levels of metals to the media, but these are present at final concentrations comparable to standard tap water.
- The level of salt currently used to induce sugar production in these cyanobacteria (100-200mM) corresponds to ~0.6 to 1.2% salt. Marine environments have salt concentrations between 3-5%. We anticipate that the cost of adding salt to a large-scale production system could be diminished through the use of filtered marine, or brackish, waters. However, another possibility to reduce the need for salt would be to genetically alter the cyanobacteria to cause it to produce sucrose even in the absence of salt. We are currently investigating this possibility.
- CscB-expressing *S. elongatus* grows more slowly than wild-type cyanobacteria. The production and subsequent export of sucrose means that the cells are diverting resources towards a product that they ultimately do not have access to for growth. Therefore, the more active the

cscB-dependent sugar pathway is, the slower the growth of the cyanobacteria (Figure 4). It is unlikely that this strain would survive well in the wild, as it would be rapidly outcompeted by other organisms (including wild-type cyanobacteria).

- Species within the larger genus *Synechococcus* account for a great deal of the natural cyanobacterial species in marine and freshwater environments. Indeed, by some estimates *Synechococcus* may account for up to 25% of the primary productivity in marine environments.
- Although some species within the *Synechoccus* genus produce protective compounds that can be toxic, *S. elongatus* is not known to produce any natural toxins.

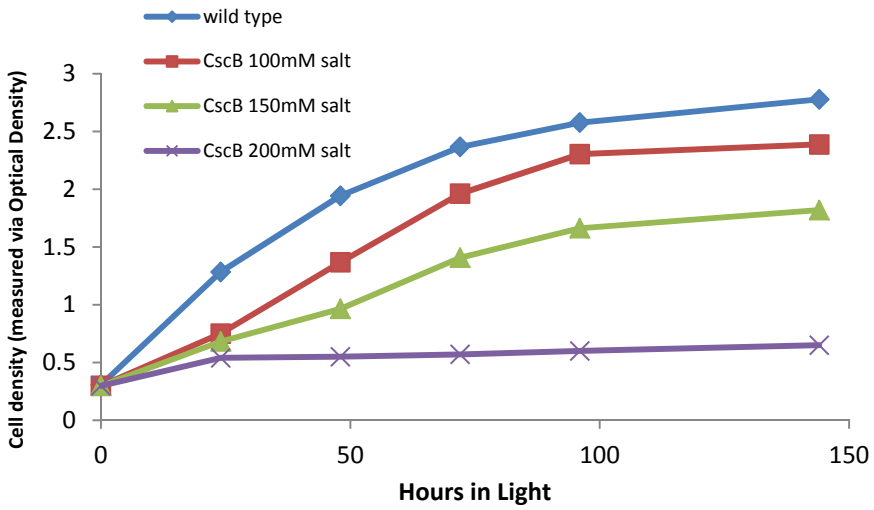

Figure 4. Cell growth in response to CscB expression. Cultures of wild-type, or CscB-expressing cyanobacteria are monitored for growth as a function of increasing optical density. The higher the concentration of salt used, the more sucrose is exported, and the slower the growth of the cyanobacteria.

Growing cyanobacteria at industrial scales

The primary feedstocks of engineered cyanobacteria are carbon dioxide and sunlight. This makes them an attractive platform for the sustainable production of biofuels and other chemicals, but it also requires the growth of engineered bacteria in an outdoor environment. Economically feasible, large-scale cyanobacteria production requires large surface areas for photosynthesis. Genetic modification is required to attain high product yields. These two factors run counter to the design of engineering controls to prevent the release of genetic material into the environment.

Currently, most large-scale cyanobacteria growth facilities are of an open-pond design (Figure 5). This is the least expensive method of growing large amounts of natural cyanobacteria. Cyanobacteria that are engineered to produce large amounts of a chemical such as sucrose, however, grow more slowly than natural cyanobacteria and are therefore unsuitable for open pond growth. Minimal containment (e.g., photobioreactor systems using plastic bags containing the engineered strain—Figure 6) will likely be necessary to prevent natural cyanobacteria from contaminating the facility.

Minimal containment may also be economically viable, but any containment solution must also allow gas exchange for carbon dioxide uptake. Bioreactor complexity is problematic because of the large surface area required for growing cyanobacteria. For example, a materials cost of $5 per square meter equates to a capital cost of $20 million for a 400-hectare facility.

Figure 5. An open-raceway pond designed for the growth of wild-type *Spirulina pacifica*. Source: Nutrex Hawaii.

Figure 6. Example of a photobioreactor facility for producing algae and cyanobacteria.
Photo from a South African biofuel project. www.saaea.blogspot.com

Producing higher-value commodities can mitigate the costs of containment. We estimate our current system for sucrose production may be capable of producing 14 tons of sucrose per hectare per year. At a sucrose price of $0.50 per kg,[3] a 400-hectare operation would be worth $2.8 million USD per year, or $7,000 per hectare. In comparison, it costs about $563 per hectare to produce $966 worth of corn[4]. If operating costs for sucrose production are to be comparable to corn (~58%), operating costs should aim to be around $4,100 USD per hectare per year. Fully enclosed photobioreactors have been assessed to cost upwards of $15,000 USD per hectare per year[5].

To maximize the economic feasibility of cyanobacteria scale-up, it will be important to anticipate containment issues and solve them via strain engineering. A key advantage of engineering biological systems is that they are self-replicating; improving a microbial strain is often a one-time cost. Engineering controls, on the other hand, scale with the size of the facility. In the long term, it is better to design microbial strains to be safe than to build extra containment at each site.

How can cyanobacteria be engineered to be safer? One approach is to make the host strain unfit for life outside the growth facility. Well-engineered strains will be less fit than their wild-type counterparts due to the redirection of metabolic flux from growth and maintenance to the production of the desired compound. We envision that cscB-expressing cyanobacteria will divert upwards of 95% of the carbon they fix to sucrose production. In the case of accidental release of the engineered cyanobacteria into the environment, it is likely that wild strains of bacteria will quickly outgrow the engineered strain. This factor also creates an economic incentive for containment rather than against it, as the engineered strain will need to be protected from outside species.

One method of physical containment is to maintain growth pond conditions that are incompatible with the surrounding flora and fauna. For example, many cyanobacteria strains are capable of living in high salinity, high temperature, or extreme pH environments. Growing a high-salt tolerant strain of cyanobacteria surrounded by a freshwater ecosystem could prevent engineered and natural microbes from interacting. A potential problem with this approach is the physical damage that could occur from a loss of containment; release of high-salt water could harm the surrounding area. Experience with salt evaporation ponds (Figure 7), which naturally accumulate salt-tolerant cyanobacteria, suggest that these risks can be managed.[6]

Figure 7. Salt evaporation ponds outside San Francisco. Pond salinity levels can be distinguished by the different cyanobacterial species that grow in them. Containment is provided by levees surrounding the ponds.

[3] Ducat et al. Engineering cyanobacteria to generate high-value products. Trends Biotechnol (2011) vol. 29 (2) pp. 95-103

[4] http://www.ers.usda.gov/publications/sb974-1/sb974-1.pdf

[5] http://www.dotyenergy.com/Markets/Micro-algae.htm

[6] http://www.cargill.com/cs/sf_bay/

In the event of a release of engineered cyanobacteria into the environment, growth of the engineered strain in the wider environment is not the only risk. Uptake of genetic material by wild bacteria may transfer new gene functions into the ecosystem. For this reason, industrial cyanobacteria should not include any antibiotic resistance genes that are often used during strain construction. Engineered strains should also be designed to be free of toxins or disease-related genes.

Managing the safety of heterologous genes in engineered cyanobacteria is more difficult. In the case of sucrose-producing cyanobacteria, more efficient sucrose-producing enzymes and sucrose transporters may be beneficial to other bacterial strains. A further complication is that engineered genes are often grouped together in the host genome. Grouping genes simplifies the design and troubleshooting process, but also makes it easier for several engineered genes to be horizontally transferred in a single event. Furthermore, it can be difficult to predict how engineered genes will behave; it may be impossible to fully determine how engineered genes will affect other bacteria.

At an industrial scale, two conflicting factors must be reconciled: economic demands to reduce capital costs, and the need for safe containment of engineered organisms. Both issues can be partially addressed through better strain design, but a more thorough risk assessment is required to identify strain parameters to improve. If these risks can be managed, engineered cyanobacteria may be able to provide fuels, high-value compounds, and biomaterials in a carbon-neutral manner.