Report

Background

Metabolic Engineering

An emerging approach to the understanding and utilization of metabolic processes is Metabolic (or pathway) Engineering (ME). As the name implies, ME is the targeted and purposeful alteration of metabolic pathways found in an organism in order to better understand and utilize cellular pathways for chemical transformation, energy transduction, and supramolecular assembly. ME typically involves the redirection of cellular activities by the rearrangement of the enzymatic, transport, and regulatory functions of the cell through the use of recombinant DNA and other techniques. Much of this effort has focused on microbial organisms, but important work is being done in cell cultures derived from plants, insects, and animals. Since the success of ME hinges on the ability to change host metabolism, its continued development will depend critically on a far more sophisticated knowledge of metabolism than currently exists.

This knowledge includes conceptual and technical approaches necessary to understand the integration and control of genetic, catalytic, and transport processes. While this knowledge will be quite valuable as fundamental research, per se, it will also provide the underpinning for many applications of immediate value.

Scope

The Metabolic Engineering Working Group is concerned with increasing the science and engineering community's level of knowledge and understanding of ME. The Working Group strives to encourage and coordinate research in ME within academia, industry, and government in order to synergize the Federal investment in ME.

Introduction

In November 1995, Science Advisor John H. Gibbons of the Office of Science and Technology Policy (OSTP) released the report, "Biotechnology for the 21st Century: New Horizons." This report was a product of the Biotechnology Research Subcommittee (BRS) under OSTP, and identifies priorities for federal investment and specific research

opportunities in biotechnology. These priorities include agriculture, the environment, manufacturing and bioprocessing, and marine biotechnology and aquaculture. The BRS formed several working groups to facilitate progress on some of these key priorities. The Metabolic Engineering Working Group (MEWG) was created to foster research in Metabolic Engineering, an endeavor that can contribute to all of the key priorities in the aforementioned report. The Working Group is composed of Federal scientists and engineers who participate as part of the activities of OSTP, and represent all of the major agencies involved in Metabolic Engineering research.

In its on-going efforts to promote and enhance the use of Metabolic Engineering (ME), the Working Group sponsored its fourth annual interagency workshop of federal scientists and engineers associated with ME research. This workshop was held May 31, 2000 at the National Science Foundation, in Arlington, VA. The purpose of the workshop was to showcase the grantees from the first the Interagency Announcement of Opportunities in Metabolic Engineering, which was issued in January 1998. In addition, an afternoon session was devoted to a better understanding of bioinformatics and metabolic engineering, chaired by topic experts. The workshop was designed to be a tutorial for agency representatives and decision makers that participate in reviewing and funding proposals in related disciplines. The Metabolic Engineering Working Group has since issued a second Announcement of Opportunities in Metabolic Engineering and selected research grantees for 1999 - 2000.

Purpose of Workshop

The purpose of the fourth Interagency Workshop on Metabolic Engineering was to:

> Educate Federal agency personnel on emerging metabolic engineering issues through presentations by experts in the field, and highlight the progress of the grantees from the first Interagency Announcement.
>
> Discuss possible topic areas of interest amongst the agencies for future Interagency Announcements on Metabolic Engineering.

The Scope of the 1998 Interagency Announcement

Three topic areas were specified in the FY 1998 Interagency Announcement and awards were made in each area. The topics were:

> Instrumentation, sensors, new analytical tools, and new cell and molecular biology methods which facilitate the study of metabolic pathways, especially those technologies that allow the examination of individual cells.
>
> Quantitative and conceptual models integrated with experimental studies that better characterize the regulation and integration of complex, interacting metabolic pathways.
>
> The use of bioinformatics to deduce the structure, function, and regulation of major metabolic pathways from the genomic sequence data bases.

1998 Interagency Metabolic Engineering Grants

Principal Investigators	Institute	Title	Award Amount	Award Time Period
L.O. Ingram, J.F. Preston, & K.T. Shanmugam,	University of Florida	Advanced Ethanologenic Biocatalysts for Lignocellulosic Fermentations	$498935	FY1998-FY2000
Michael J. Betenbaugh	Johns Hopkins University	Carbohydrate Engineering for Generating Sialylated Glycoproteins in Insect Cells	$814706	FY1998-FY2000
Andrew D. Hanson	University of Florida	Engineering Plant One-Carbon (1-C) Metabolism	$268000	FY1999-FY2001
Bernhard Palsson	University of California-San Diego	In Silico Analysis of the Escherichia Coli Metabolic Genotype and the Construction of Selected Isogenic Strains	$274593	FY1999-FY2001
Bernhard Palsson	University of California-San Diego	Computational Infrastructure for Engineered Microorganisms	$850000	FY1998-FY2000
Bernhard Palsson	University of California-San Diego	Genomically Based Models for Antimicrobial Development	$680957	FY1998-FY2000
Mary E. Lidstrom, Steven Van Dien	University of Washington	Metabolic Engineering of Methylotrophic Bacteria for Conversion of Methanol to Higher Value Added Products	$380000	FY1998-FY1999
Jay D. Keasling	University of California-Berkeley	Strategies for Metabolic Engineering of Environmental Microorganisms - Application to Degradation of Organophosphate Contaminants	$397749	FY1999-FY2001

Abstracts of Expert Presentations

ENGINEERING PLANT ONE-CARBON METABOLISM

Andrew Hanson
University of Florida

Primary and secondary metabolism intersect in the one-carbon (C_1) area, with primary metabolism supplying most of the C_1 units and competing with secondary metabolism for their use. This competition is potentially severe because secondary products such as lignin, alkaloids and glycine betaine require massive amounts of C_1 units. Many current metabolic engineering projects aim to change levels of these products, or entail reducing the supply of C_1 units. It is therefore essential to understand how C_1 metabolism is regulated at the metabolic and gene levels so as to successfully engineer C_1 supply to match demand. Our project aims to acquire this understanding. Specific objectives are: (1) to clone complete suites of C_1 genes from maize and tobacco, and to incorporate them into DNA arrays; (2) to use sense and antisense approaches as well as mutants to engineer alterations in C_1 unit supply and demand; and (3) to quantify the impacts of these alterations on gene expression (using DNA arrays), and on metabolic fluxes (by combining radio- and stable isotope labeling, MS, NMR and computer modeling).

Four findings from Year 1 will be summarized. All were unexpected and have implications for engineering C_1 metabolism: (1) Unlike other eukaryotes, plants have methylenetetrahydrofolate reductases that use NADH rather than NADPH as reductant, and are not allosterically inhibited by AdoMet. (2) DNA arrays show that formate dehydrogenase and a cluster of enzymes for methyl group synthesis and transfer are more highly expressed in roots than leaves. (3) Metabolic flux analysis and modeling of tobacco engineered to convert choline to glycine betaine suggests a crucial role for a chloroplast choline transporter. (4) Plants have an unsuspected source of formate B the irreversible hydrolysis 10-formyltetrahydrofolate, via an enzyme previously known only in prokaryotes. The first and last of these findings depended on genomics-based approaches, and illustrate the value of bioinformatics in metabolic engineering.

CARBOHYDRATE ENGINEERING FOR GENERATING SIALYLATED GLYCOPROTEINS IN INSECT CELLS

Michael J. Betenbaugh
Johns Hopkins University

Insect cells are used to generate of a variety of biotechnology products. Many of the most valuable biotechnology products are glycoproteins that include oligosaccharides attached to the protein at particular amino acids. Unfortunately, processing in insect cells yields glycoproteins with different carbohydrate structures from those generated by human and other mammalian hosts. While mammalian cells produce complex oligosaccharides often terminating in the sugar, sialic acid, insect cells typically generate simpler oligosaccharide structures. Since these covalently attached carbohydrates can significantly affect a protein's structure, stability, biological activity, and *in vivo* circulatory half-life, the objective of this project is to manipulate carbohydrate-processing pathways in insect cells to generate complex humanized glycoproteins terminating in sialic acid. The sialylation reaction involves the addition of a donor substrate, cytidine monophosphate-sialic acid (CMP-SA or CMP-NeuAc), onto a specific acceptor carbohydrate via an enzymatic reaction in the Golgi apparatus. Therefore, each of the three reaction components, donor substrate,

acceptor substrate, and enzyme, must be engineered into insect cells using metabolic engineering strategies. Production of the donor substrate, CMP-SA, will be achieved by adding key metabolic precursors such as N-acetylmannosamine (ManNAc) to the growth media and by genetically manipulating insect cells to express limiting enzymes in the CMP-SA production pathway. These genes have been obtained from known mammalian sequences or identified using homology searches of known bacterial sequences. The generation of correct carbohydrate acceptors is achieved by suppressing unfavorable cleavage reactions and by enhancing the expression of favorable glycosyltransferase enzymes such as galactose transferase. The completion of the sialylation reaction will be obtained by expressing the catalyzing sialyltransferase enzyme in the presence of these correct acceptor and donor substrates. Engineering the sialylation reaction into insect cells may increase the value of insect cell-derived products as vaccines, therapeutics, and diagnostics. Humanizing insect cells and other recombinant DNA hosts will make expression systems more versatile and may ultimately lower biotechnology production costs. In the future a particular host may be chosen based on its efficiency of production rather than its capacity to generate particular oligosaccharide profiles.

APPLICATION OF FUNTIONAL GENOMICS TO THE DEVELOPMENT
AND OPTIMIZATION OF BIOCATALYSTS FOR RENEWABLE FUELS AND CHEMICALS

Lonnie O. Ingram
University of Florida

Today, the genetic piping of metabolic pathways to fuel ethanol and higher value products such as aromatics or plastics, and the hyper-expression of recombinant proteins in cells as factories for biotransformations offer the potential to replace imported petroleum with renewable biomass. Previous USDA and DOE awards have supported the development of recombinant *Escherichia coli* K011, an organism capable of producing ethanol efficiently from monomers of all carbohydrate constituents in lignocellulose. Subsequent awards developed recombinant *Klebsiella oxytoca* P2 for cellulose bioconversion, eliminating the need to externally supply _-glucosidase. Current funding has extended cellobiose utilization to recombinant *E. coli,* and engineered the expression and secretion of high levels of *Erwinia* endoglucanases in both organisms. Studies funded by the Metabolic Engineering Working Group (USDA-NRI & DOE-BES) have improved our removal of toxins generated during dilute acid hydrolysis pretreatments of biomass, moving toward a simplified process. With the completion of the *E. coli* genome, the most widely used microbial platform for the new biotechnology, it is now time to apply Functional Genomics to these problems. Continuing research will focus on the molecular tuning of biocatalysts to maximize resistance to toxins and ethanol, to increase rates of glycolytic flux, and to reduce the time required for the completion of bioconversions. Initial studies have investigated the expression of the entire *E. coli* genome during model fermentations of 100 g/L xylose to 50 g/L ethanol. Initial examination of these data have provided a wealth of new information concerning the isoenzymes used in central pathways, changes in gene expression responsible for increased flux, clues to genes involved in ethanol tolerance, evidence for unexpected co-regulation of central metabolism genes, etc. Our initial Transcriptome data has facilitated the development of many hypotheses that can be readily tested using expression vectors and chromosomal mutations. Although we have none as yet, the development of companion information concerning the Proteome by 2-D gel analysis or other methods would further enhance the uitility of the data.

Transcriptome and Proteome data should be used in the near term to guide the molecular tuning of recombinant biocatalysts for renewable fuels and chemicals. This Functional Genomic data should be shared with the research community in publications and on the WWW. Results from this type of work could serve as a base for a variety of biotransformation processes, and for the improved utilization of genomic information from other organisms in biotechnology applications.

METABOLIC ENGINEERING OF METHYLOTROPHIC BACTERIA FOR CONVERSION OF METHANOL TO HIGHER VALUE-ADDED PRODUCTS

Mary Lidstrom
University of Washington

Methanol is an attractive possibility as an alternative to petroleum as a chemical feedstock. It is relatively inexpensive, soluble in water, and since it is produced from methane, it is a renewable resource. Methylotrophic bacteria are capable of growth on one-carbon compounds, such as methanol. As such, they represent the potential to convert methanol to a variety of potential products. In order to manipulate methylotrophic metabolism, metabolic engineering will be required, both to understand methylotrophy in more depth and to alter the flow of carbon from methanol to desired products. Methylotrophs are actually growing on formaldehyde, their key intermediate for both carbon and energy metabolism. Therefore, the key to manipulating methylotrophic metabolism in methylotrophs is to understand formaldehyde handling. The organism of choice for this project is *Methylobacterium extorquens* AM1, an _-proteobacterium that grows on methanol, methylamine, and a variety of multi-carbon compounds. It is already known that about 75 gene products are involved in methylotrophic metabolism in this organism, a substantial toolkit of genetic capabilities are available, and in collaboration with the UW Genome Sequencing Center, an unfinished genome sequencing project is in progress. We have initiated metabolic engineering studies by examining the pathway of polybetahydroxybutyrate (PHB) synthesis. Not only is this polymer a potential target product, it is a major part of the biomass generated during regular growth on methanol. Therefore it is important to understand it=s role in overall metabolism during growth on methanol. We have cloned the genes for PHB synthesis and degradation using information from the genome sequencing project, and have generated mutants in these genes. Surprisingly, mutants in PHB synthesis are unable to grow on methanol. Further analysis has suggested that D-betahydroxybutyrate, the precursor to PHB synthesis, is an intermediate in a part of the serine cycle that involves the conversion of acetylCoA to glyoxylate. We are currently examining the role of NADPH/NADH ratios in methylotrophic metabolism, using a combination of metabolic modeling and metabolic engineering.

STRATEGIES FOR METABOLIC ENGINEERING OF ENVIRONMENTAL MICROORGANISMS: APPLICATION TO DEGRADATION OF ORGANOPHOSPHATE CONTAMINANTS

Jay D. Keasling
University of California, Berkeley

Biodegradation of readily-degradable contaminants has proven to be an effective treatment strategy for environmental restoration. Although bacteria have the capacity to transform a number of chemicals, many compounds have novel structures or substituents rarely found in nature and are recalcitrant to biodegradation or are extremely toxic. To expand the range of compounds that can be degraded by biological systems, we must assemble the appropriate enzymatic reactions to catalyze the transformations, either by introducing the genes for the enzyme-catalyzed reactions into a single bacterium or by assembling a consortium of bacteria containing one or more of the necessary enzymes to undertake the transformation. Analogous to the design of chemical manufacturing facilities, the flow of chemicals through enzymatic reactions within a cell must be optimized within the context of other cellular processes to ensure that the toxic compound is fully degraded, to minimize the generation of undesirable products, and to ensure that the engineered organism can compete in the environment.

Advances in molecular biology have given rise to a number of tools to manipulate gene expression. However, most of

these tools have been developed for overproduction of pharmaceutical proteins and, as such, have not been optimized for metabolic engineering of environmental organisms. Furthermore, there are few technologies available to coordinately regulate multiple, heterologous, biodegradation pathways in a single organism, particularly for degradation of a contaminant.

The goal of this work has been to develop the experimental and theoretical methods to introduce multiple, heterologous, biodegradation pathways into a single organism and to optimize the flux through those pathways for the remediation of toxic or recalcitrant organic contaminants. This work focuses on the biodegradation of the organophosphate contaminants by an engineered strain of *Pseudomonas putida*. We have chosen parathion as a model compound because (*i*) it has been widely used as a pesticide, (*ii*) it could potentially serve as a source for carbon, phosphorus, and sulfur for cell growth, (*iii*) no single organism has been isolated that can use it as a sole carbon and phosphorus source, and (*iv*) it is similar in structure to a number of other important environmental contaminants, such as nerve agents and other pesticides, but is relatively safe for use in the laboratory. In addition, the initial enzyme in parathion degradation (parathion hydrolase), which hydrolyzes parathion to *p*-nitrophenol (PNP) and diethyl thiophosphate (DETP), has been shown to hydrolyze many organophosphate contaminants. We have chosen a well-studied species of the common soil bacterium *Pseudomonas*, *Pseudomonas putida*, as its genetics and metabolism have been well characterized.

The specific aims of this work are as follows: (*i*) to develop a flux-based, metabolic model for *Pseudomonas putida* to predict necessary fluxes through the native and heterologous pathways for optimal growth and biodegradation; (*ii*) to clone the genes for DETP degradation from *Comomonas acidovorans*; (*iii*) to place the genes for the enzymes involved in DETP degradation in an operon; (*iv*) to introduce into *P. putida* the gene encoding parathion hydrolase and the operons of genes responsible for PNP and DETP degradation; (*v*) to coordinate expression of the *opd* gene and the PNP and DETP operons at the optimal levels for maximal growth and biodegradation rates.

Although this work has focused on the degradation of parathion, we anticipate that the technologies developed here will be applicable to the degradation of other organophosphate contaminants, such as nerve agents, and recalcitrant organic contaminants. The development of rational metabolic engineering technologies for environmental restoration will lead to improved degradation rates, more complete degradation of the contaminant, and bacteria that can compete better in the environment. The application of biodegradation to treat extremely toxic contaminants, such as organophosphate nerve agents, may necessitate such strategies.

IN SILICO ANALYSIS OF THE *ESCHERICHIA COLI* METABOLIC GENOTYPE AND THE CONSTRUCTION OF SELECTED ISOGENIC STRAINS

Bernhard Palsson
University of California, San Diego

During the first year of this program we have made significant progress towards the stated goals.

Goal #1: Automate the generation of metabolic genotypes

Task 1: We have constructed a new *in silico* metabolic genotype for *Haemophilus influenzae* Rd (strain KW20) and have nearly completed a model for *Helicobacter pylori* (strain 26695). The genes present in each genome were

determined using genomic microbial databases accessible on the Internet, as well as published biochemical data. A list of the genes present is compared to our database of known metabolic reactions (initiated with our *in silico E. coli* K-12 strain) for the purpose of (1) associating each gene in the list with one or more reactions catalyzed by the gene as found in our database; and (2) expanding our database to accommodate more reactions. The corresponding stoichiometric matrices are being formed for each new strain and used in flux-balance and pathway analysis.

Task 2: Information for the construction of a physiological database has been gathered in a literature survey focusing on *E. coli* K-12, *H. influenzae* Rd KW20 and *H. pylori* 26695. We have also performed experiments with *E. coli* K-12 in our laboratory, using glucose, succinate and acetate as carbon sources for the purpose of determining more strain-specific parameters.

Task 3: The FBA program was developed to give both quantitative and visual results to the linear optimization of a given objective function, most commonly growth. The program calculates traditional linear programming values such as the objective function and shadow prices. The results are then graphically displayed in pathway form with the corresponding fluxes labeled for each reaction and the utilized pathways highlighted.

Task 4: This task has not yet been addressed.

Goal #2: Develop methods to determine genotype properties and capabilities

Module 1: The FBA program that we developed allows for the deletion of any number of genes. The corresponding phenotype can than be analyzed *in silico*. A metabolic model of *Haemophilus influenzae* has been developed by our group and will be used to do deletion analyses similar to those done in *E. coli*. In addition, the FBA program has been used to do robustness analyses in *E. coli.* Such an analysis examines the sensitivity of the growth function to altered flux levels of essential genes that have been identified in the central metabolic pathways.

Module 2: The FBA program is designed to plot Phenotypic Phase Planes (PhPP), which are a phenotypic mapping for biomass generation as a function of a primary carbon source and oxygen uptake rate. These PhPPs show the metabolic shifts that occur with various oxygenation and substrate levels. Experiments have begun in the lab to verify the metabolic shifts predicted using the *in silico* metabolic model for *E. coli*.

Module 3: This module is in progress.

Panel Discussion

The afternoon session consisted of a panel discussion lead by George Church and Bernhard Palsson.

The workshop focused on the Metabolic Engineering program and desirable changes in its direction. It has become clear that the emphasis of this program will become the use of microbial genetics and systems analysis methods to attempt to synthesize mechanistic description of the genotype-pheotype relationship; or in other words to go from genomics to phenomics.

This grand challenge involves the development of instrumentation, data basing, algorithm development, and model

formulation. These issues were discussed on a wide basis and all opinions were heard.

Issues raised and focused recommendations:

A. Data generation (to drive computation work):

A.1) More quantitative data is needed. Where the costs of this could benefit greatly from new instrumentation encourage clear communication with the appropriate engineering groups.

A.2) Generate ways to estimate/catalog physico-chemical properties of protein (enzymes in particular).

A.3) Make funding for arrays available once sequences are established especially since this is a small fraction of the sequencing cost.

A.4) Instrumentation for high-throughput phenotyping is needed. Desired phenotypic data include: growth rates of cells and organisms, RNA, protein and metabolite assays on single cells and populations.

B. Databases and data sharing.

B.1) It was observed that purely database creation grants have not received high marks during peer review. Applicants should be encouraged to couple such databases with creative goals, methods, and/or models.

B.2) Standardization is needed for both software and data and both syntax, semantics.

B.3) Encourage deposit of computationally parseable versions of data generated under program where it is retrievable (e.g. www). As needed, design databases to connect new data types with new applications.

C. Models, Software and Algorithms:

C.1.) Algorithm and model development is good, but more software is needed. Models need to be (at a minimum) reproducible by experts by a simple download and run.

C.2) Point-and-click web accessible software is needed for more general use.

C.3) Standardization/portability is needed for math models.

C.4) Biology is stated by many to be too complex for math analysis, but the converse would be stated by systems scientist, namely that it is hard to understand such complex processes without a model.

C.5) What is the accuracy available (false negative and positive rates) for pathways generated de novo in silico?

Encourage estimates of the costs of improving the accuracy.

C.6) Encourage determination of the level/accuracy of kinetic constants that are needed for good models.

D. Administrative:

D.1) Broaden announcement to include 'functional genomics'.

D.2) Microbial genomics needs to be merged into metabolic engineering.

D.3) Be careful in the use of language in announcement

- bioinformatics vs. functional genomics,
- math model vs. reconstruction,
- deduction vs. engineering,
- Physico-chemical-properties vs functional genomics.

Conclusions and Discussions of the Panel Discussion

The attendees at The Panel Discussion focused on the future needs for augmenting the bioinformatic content in the Metabolic Engineering Joint Research activity. Attendees included all of the Year 1 grantees, several of the Year 2 awardees, and about 15-20 representatives from the agencies participating in the MEWG. The *conclusions* and *recommendations* from this Discussion represent the collective opinions of the attendee group as reflected in comments made at the Discussion as well as several e-mail responses sent to one or more of the MEWG representatives.

The consensus was that the program needs to foster the development of tools to facilitate the translation of genomic information into real biological processes -- e.g directed protein synthesis and metabolism. Tools that were specifically discussed included databases, functional genomics, and nucleic acid and protein high-throughput screening methodologies. On the subject of databases, the observation was made that good metabolic models are data starved --- there is a need for genomic, proteomic and metabolomic data that is validated, widely distributed (e.g. the web-based Biology Workbench), and curated. It was also noted that data generation, accumulation and genomic-to-phenomic modeling should be done in the context of the new processes and products that can be realized by recombinant DNA technology. DNA, RNA and protein array technology should be fostered in the program, and it was noted that many of the Years 1 and 2 grantees are already using these methodologies and in several cases making important contributions to the advancement of these tools. Finally, opinions were widely voiced that modeling work needs to be tightly coupled to experimental effort, and that the program should be kept appropriately broad so that opportunities for investigator-driven research are maximized.

AGENDA

8:00 am **Welcoming and Opening Remarks**

MARYANNA HENKART, Chair, Biotechnology Research Working Group

FRED HEINEKEN, Chair, Metabolic Engineering Working Group

8:15 am **Engineering Plant C1 Metabolism**

ANDREW HANSON, University of Florida

8:45 am **Carbohydrate Engineering for Generating Sialyated Glycoprotiens in Insect Cells**

MICHAEL BETENBAUGH, Johns Hopkins University

9:15 am **Metabolic Designs to Maximize Ethanol Production from Lignocellulose**

LONNIE INGRAM, University of Florida

10:15 am **Welcoming Remarks**

MARY CLUTTER, Chair, Subcommittee on Biotechnology

10:30 am **Metabolic Engineering of Methylobacterium extorquens AM1 for Conversion of Methanol to Higher Value Added Products**

MARY LIDSTROM, University of Washington

11:00 am **Strategies for Metabolic Engineering of Environmental Organisms: Application to Degradation of Organophosphate Contaminants**

JAY KEASLING, University of California - Berkeley

11:30 am **Progress Report Grant BES 98-14092**

BERNARD PALSSON, University of California - San Diego

1:15 pm **Introduction to Afternoon Session**

Convener BERNARD PALSSON

1:20 pm **Measuring & Modeling Cellular Metabolic & Regulatory Networks**

GEORGE CHURCH, Harvard University

1:35 pm **The Needed Information Technology Infrastructure**

JOHN WOOLEY, University of California - San Diego

1:50 pm **Predicting Physico-Chemical Properties of Gene Products**

MICHAEL GILSON, National Institute of Standards and Technology

2:20 pm **Numerics and Modeling Philosophies**

LESLIE LOEW, University of Connecticut Health Center

2:50 pm **Introduction to general discussion on Bioinformatics**

MARK SEGAL, Environmental Protection Agency
VINCE VILKER, National Institute of Standards and Technology

3:05 pm **General Discussion and Future Directions**

BERNARD PALSSON, University of California - San Diego
GEORGE CHURCH, Harvard University

4:15 pm **Adjourn**

Agency Activities in Metabolic Engineering

U.S Department of Agriculture

The Agricultural Research Service (ARS) and the Forest Service (FS) conduct metabolic engineering research through

the Federal laboratory system while the Cooperative State Research, Education, and Extension Service (CSREES) supports metabolic engineering research through competitive research grants and through formula-based programs in cooperation with the states.

USDA research activities encompass animal sciences, plant sciences, commodity conversion and delivery, environmental sciences (air, soil, water), human nutrition, and integration of agricultural systems.

Metabolic engineering technologies are being developed and applied across the above research areas and include the following goals:

To modify microbial metabolism for the production of commercially useful products, chemicals, biofuels, and biomolecules from agricultural commodities and resources.

To develop genetic and other techniques for altering metabolic pathways to understand basic processes associated with microbial based natural or newly developed biocontrol agents resulting in elimination, decreased use, or increased environmental bioremediation of both agricultural wastes and agricultural chemicals such as herbicides, insecticides, fungicides, or biocides.

To improve efficiency of production and decrease losses due to environmental stresses, diseases, pathogens, parasites, or pests by altering host metabolism using genetic or other techniques to apply metabolic engineering at the tissue, organ, or whole organism level of animals or plants, alone or in combination with the microorganisms associated with these hosts.

Ongoing research includes:

Metabolic engineering for the development of superior fuel ethanol producing microorganisms. Microorganisms that normally use multiple substrates are being engineered for enhanced ethanol production, and microorganisms that normally make ethanol are being engineered to use multiple substrates.

Metabolic engineering for the development of superior solvent producing anaerobic bacteria. Specifically, the fermentative enzymes involved in butanol production are being analyzed in order to manipulate metabolic fluxes from acidogensis to solventogenesis.

Metabolic engineering of anaerobic bacteria for improved animal performance. The specific approach is to enhance xylan degradation of feed material by introducing into the rumen a genetically modified bacterium that overproduces xylanase.

Metabolic engineering of toxigenic fungi and host plants. Specifically, the genes involved in aflatoxin biosynthesis have been identified and a master switch gene discovered. By engineering plants to favor production of a metabolite that interferes with this master gene, aflatoxin production may be prevented in the host plant.

Modify metabolite distribution in plants. One specific approach is to transfer the liquid wax producing capability of jojoba into a metabolic pathway for commercially viable oilseed rape and soybeans.

National Institute of Standards and Technology

NIST has internal research programs in the Biotechnology Division, and extramural collaboratively funded research and development programs through the Advanced Technology Program that are related to the scientific field known as Metabolic Engineering. Each of these programs have different foci and management structures, but share the overall goal of fostering the commercialization of recent scientific advances in areas related to biotechnology, such as biocatalysis and metabolic engineering.

Biotechnology Division (Intramural)

In the intramural programs of the Biotechnology Division (http://www.cstl.nist.gov/biotech) , which is one of five Divisions of the Chemical Sciences and Technology Laboratory, the mission is to advance the commercialization of biotechnology by developing the scientific/engineering technical base, reliable measurement techniques and data to enable U.S. industry to quickly and economically produce biochemical products with appropriate quality control. The mission is carried out in collaboration with industry, other government agencies and the scientific community. The primary research efforts that relate to Metabolic Engineering are in Bioprocess Engineering, Structural Biology, DNA Technologies, and Biomolecular Materials groups.

The Bioprocess Engineering (http://cstl.nist.gov/div831/bioprocess) activity includes *biophysical property evaluation* where thermophysical and thermochemical properties are being obtained, evaluated, codified and modelled for biochemicals, proteins and biosolutions of interest in metabolic pathway engineering. A research program in *biocatalysis* is underway to solve technical roadblocks in the commercial development of enzymes that build new complex molecules used in advanced drug or food product design. Investigations are currently focused on finding generic routes for meeting the energy requirements of these enzymes, and on developing synthetic methods of carrying out cell functions like electron transfer between proteins.

The Structural Biology activity includes x-ray amd NMR measurements of atomic structures of prototypical proteins, enzymes, enzyme-substrate complexes and model DNA systems. A research program in *biothermodynamics* uses state-of-the-art calorimetric methods to study protein-protein and protein-substrate interactions, and computational models are developed that relate structure to function. Physical and biochemical methods are used to characterize protein behavior, including the study of membrane-embedded proteins to understand signal transduction. *Computational chemistry and modelling* develops methods to model the energetics and dynamics of interactions between substrates and active sites of enzymes. Modelling techniques to understand the relationship between protein sequence and structure are being developed.

The DNA Technologies activity includes development of methods and standards for *DNA profiling* for forensic and other uses. Research is being conducted to develop the next generation of DNA profiling based on polymerase chain reaction (PCR) technology including new methods development for rapid DNA extraction, amplification, separation, and computer imaging. *DNA sequencing* develops specific reference materials and technical expertise that are are essential for DNA Genomic research in the public and private sector. This activity also provides quality assurance expertise to the developers of technology that proposes to use DNA recognition sites on silicon chips for the diagnosis of human genetic diseases. Research on *DNA damage and repair* is developing methods to characterize DNA damage on a molecular scale using GC/MS techniques. Studies of both *in-vivo* and *in-vitro* systems are underway to

understand both damage (as low as one base per million) and repair mechanisms.

The Biomolecular Materials activity develops generic measurement technologies utilizing both optical and electrochemical approaches for applications in clinical diagnostics, bioprocessessing, and environmental monitoring. Research on lipid membranes and membrane proteins is being performed to provide an understanding of materials and methods that will enhance the development of this important class of molecules in sensor and other applications. The light-sensitive protein, bacteriorhodopsin is being studied as a potential source for the storage and retrieval of information. Studies are underway to understand and control the mechanism of this optical transition, and to develop methods of immobilizing this protein to increase its stability.

Advanced Technology Program (Extramural)

The Advanced Technology Program within NIST provides funding to support innovative research and development which are likely to lead to inventive new technologies and products that will have positive economic benefits for the United States. ATP has in the past, and continues to fund projects in Metabolic Engineering. These projects include the modification of enzymatic pathways in microorganisms and improved bioprocessing technologies to produce, in a cost-effective way, monomers used in the synthesis of thermoplastics, essential cofactors for human health, disease-targeted therapeutics and desulfurized crude oil. Support also has been provided to companies seeking to engineer the synthesis of isoprenoids in yeast and biopolymers in the fibers of cotton plants. The production of better goods at lower costs and the utilization of renewable biosystems are potential benefits to be derived from these projects. As documented in more than a dozen White Papers submitted to ATP, industries' future commitments for applications of metabolic engineering are expansive and cover wide areas including immobilized biocatalysis, novel bioreactors, value-added crops, better nutrition and an improved environment

Department of Defense

The Department of Defense (DoD) currently supports a broad range of research in the area of metabolic engineering through the Army Research Office (ARO) and other Army research activities, the Air Force Office of Scientific Research (AFOSR) and the Office of Naval Research (ONR). The specific focus of the ARO, ONR and AFOSR efforts will be summarized and future directions in metabolic engineering research and technology development will be addressed.

The broad needs for the DoD that can be served through research efforts in metabolic engineering are summarized below. These science and technology targets will provide enhanced and expanded capabilities for the missions of the services and provide greatly expanded capabilities for the civilian sector.

- Materials

- Processes

- Devices

- Fabrication Schemes

- Information Processing

Current interests in metabolic engineering at ARO are focused on two related topics: the characterization of biochemical pathways and enzymatic mechanisms and the genetic manipulationof protein structure and function. The goal is to develop a detailed understanding of how macromolecules have been tailored to execute their designated functions and how they interact with other macromolecules. With this information, it will be possible to engineer enzymes and metabolic pathways to exhibit a set of specific functions and properties, according to Army needs. ARO currently supports research in several areas, including: how molecular transport, subcellular compartmentalization, and reaction sequences are involved in enzymatic regulation and superstructural formation; understanding and manipulating aminoacylation of tRNAs to produce, using cellular translation machinery, new polymeric peptide materials containing non-natural amino acids; the role and regulation of "stress" proteins differentially expressed in response to environmental or external stimuli; and the design and implementation of unique enzymatic strategies for the biodegradation of environmental pollutants.

The AFORS focus on environmental biotechnology for cleanup and detoxification of hazardous chemicals requires considerable emphasis on the use of metabolic engineering. Many strains of microorganisms can readily use natural organic compounds such as jet fuel and gasoline as their source of carbon and energy. Such microorganisms provide the basis for the extensive recent successes in bioremediation. Metabolic engineering offers the potential for development of strains able to degrade such pollutants under adverse conditions such as in the presence of heavy metals, at elevated temperatures, or at extremes of pH.

AFOSR researchers are also using metabolic engineering in the development of microorganisms able to use synthetic organic compounds including nitro- and chloro-substituted compounds as growth substrates. Finally, a variety of reactions capable of detoxifying hazardous chemicals are known to be catalyzed by microorganisms that are unable to use the chemicals as growth substrates. Such processes can be effective for treatment of contaminants if the appropriate microbes can be stimulated to produce the necessary enzymes and confactors to sustain the reactions. Metabolic engineering offers the potential for uncoupling the production of the enzymes from the growth of the organisms. Researchers are currently developing constitutive strains with altered surface properties to allow transport in the subsurface or adherence to substrates in bioreactors. Future applications of metabolic engineering will involve the construction of strains able to degrade or synthesize a wide variety of materials relevant to not only the military, but also civilian applications.

The current ONR program provides a broad base in funding of research that addresses fundamental issues associated with metabolic engineering and, at the same time, targets a niche which is under represented in the other DoD services and in other agencies. A significant portion of the ONR program targets marine organisms as cellular factories for metabolic engineering and exploits many of the novel and unique features of marine bacteria and algae to fabricate nanostructures in which composition and shaped are defined simultaneously. Current program activities address the use of combinatorial approaches for the development of (1) biosensor devices and whole-cell biosensors, (2) new macromolecular materials, (3) novel processes and catalysts, (4) molecular composites and, (5) designer fabrication schemes. ONR is addressing the role of extracellular enzymes as catalysts for bioremediation, fabrication and for immobilized synthetic activities. In addition enzymes are being engineered to perform in non-aqueous environments which will be critical to materials synthesis, biosensor technologies, bioremediation and other critical Navy and DoD applications. Future directions include the production of proteins that serve in non-metabolic transformations such as those proteins functioning in cellular information processing, including signaling cascades, protein-based circuits and metabolic switching in which multiple metabolic pathways are coupled. Lastly, future activities will also target multi-

enzyme complexes that are coupled to generate novel structures and capabilities like those involved in polyketide synthesis.

U.S. Department of Energy

The Department of Energy is supporting over $25 million in metabolic engineering research, largely through the offices of Energy Research (ER), Energy Efficiency and Renewable Energy (EE), and Environmental Management (EM). The research falls in two main categories: 1) basic research, which involves the advancement of metabolic engineering fundamental knowledge and capabilities, and 2) applied research, which employs metabolic engineering techniques in development of target products. The basic research efforts of the Department reside within ER, whereas most of the applied research in this area is conducted within EE. In general, these research efforts are conducted by universities, national laboratories, and industry.

The Department's goals related to metabolic engineering research are to:

To expand the level of knowledge and understanding of metabolic pathways and metabolic regulatory mechanisms related to the development of novel bio-based systems for the production, conservation, and conversion of energy.

Apply metabolic engineering techniques to enhance and develop plants and microorganisms for use in the production of chemicals and fuels or for environmental remediation of waste sites.

Metabolic engineering research within ER is supported predominantly through the Office of Basic Energy Sciences (BES) and Health and Environmental Research (OHER). Most of BES's metabolic engineering research resides within the Division of Energy Biosciences. The mission of the Division is to generate the fundamental knowledge required for the development of novel bio-based systems for the production, conservation, and conversion of energy. A significant part of the program has been and continues to be aimed at the development of metabolic engineering capabilities related to plants and fermentative microbes. These activities include defining metabolic pathways, characterization of the catalytic properties of enzymes, determining metabolic regulation mechanisms, development of gene transfer capabilities, kinetic analysis of the flow through a pathway, and in a few instances the actual metabolic engineering of specific pathways. The program focuses on the development of basic scientific knowledge as opposed to the development of specific processes.

The metabolic engineering research within OHER resides in three divisions: Health Effects and Life Sciences Research, Medical Applications and Biophysical Research, and Environmental Sciences. Most of the research is conducted in association with the human genome, microbial genome, structural biology, and environmental remediation programs. OHER's research in this area is directed toward enhancing fundamental knowledge of metabolic pathways and addresses the development of tools and capabilities to elucidate the kinetics and mechanisms of microbial metabolic pathways; to create useful pathways for biotransformation of metals for biodegradation of toxic organics; and to understand complex relationships between genes, the proteins they encode, and the biological functions of these proteins in the whole organism.

In complement with its core research efforts, ER is conducting joint research with EM in support of their environmental restoration efforts and with EE in support of their fuels and chemicals production efforts. These newly formed partnerships demonstrate the spirit of collaboration and coordination within the Department, which combines science

with technology to fulfill DOE's research missions.

Metabolic engineering research within EE is supported through the offices of Transportation Technologies (OTT), Industrial Technologies (OIT), and Utility Technologies (OUT). As applied R&D efforts, the focus is on specific research and market issues within the purview of the respective office. For example, research in OTT focuses on ethanol production using bacteria and yeast that feed on sugars derived from non-agricultural feedstocks. In OIT, the focus is on the development of bioprocesses and new chemical synthesis routes using whole organisms or enzymes in the production of chemicals and materials. Finally, the research in OUT focuses on the use of photosynthetic microorganisms, such as cyanobacterium or alga blue or green algae in the production of hydrogen. In each of these program efforts, the R&D activities address metabolic engineering to increase the production of the product(s) desired by either enhancing existing pathways, constructing new pathways, or designing alternative pathways.

Environmental Management (EM) has a modest biotechnology research effort in support of its mission in waste management related to the clean-up and restoration of the U.S. national laboratory sites. The focus of this research involves bioremediation, including intrinsic, chemical bioaugmentation, and phytological approaches to clean-up chlorinated compounds, heavy metals, and other hazardous organics. Metabolic engineering approaches are being used to improve the effectiveness and efficiency of their environmental clean-up efforts by enhancing, augmenting, or creating new metabolic pathways within target organisms or plants. More recently, EM has teamed with OER to pursue basic research needs in various areas of national laboratory clean-up issues and waste management.

The biological research activities of the Department are monitored and coordinated through the BioEnergy Coordinating Committee (BECC). BECC is an interagency committee open to all organizations involved in bio-energy research and development. The committee is comprised of about 45 representatives from seven agencies and meets on a quarterly basis. The objectives of BECC are to 1) achieve effective coordination of DOE's bio-energy R&D; 2) assure optimum use of DOE's existing expertise in bio-energy R&D; 3) provide a resource for industry and others to access information rapidly in DOE bio-energy programs; and 4) achieve rapid communication within DOE of new developments, opportunities, and problems in bio-energy research and technology development.

Environmental Protection Agency

Developing Metabolic Engineering Strategies

The mission of the Environmental Protection Agency is to protect human health and the environment from adverse effects of anthropogenic activity. A prominent concern is the introduction of chemicals to the environment which may have detrimental effects on humans and other biota. As mandated by Congress, the Agency routinely conducts evaluation of chemicals intended for use, currently in use, or determined to exist at significant levels in the environment. From these evaluations, the Agency may decide to implement management strategies designed to limit the potential for adverse effects. To fulfill its congressionally mandated responsibilities, the Agency dedicates a significant portion of its resources to the development of risk assessment tools.

Coordinately, the Agency, through its stated mission as well as the implementation of congressional initiatives, such as the Pollution Prevention Act, has initiated development of technologies which 1) use chemical substitutes that are less toxic; 2) produce more efficient activity resulting in decreased requirement for the chemical or; 3) develop engineering procedures which produce little or no toxic end products. Finally, consistent with the pollution prevention ethic is the reevaluation of chemical stewardship from one of "cradle to grave" to a more multigenerational philosophy in which a

chemical may be utilitized successively in different forms prior to final disposal.

While it is generally accepted that chemical-based technologies have evolved to provide a higher standard of living for the general population, it is also recognized that the use of some chemicals, either through the chemical characteristics or the handling, synthesis or disposal, have produced negative effects on human health and/or the environment. Advances in technology allow scientists to better predict the potential for adverse effects from exposure to chemicals as well as mechanisms to diminish the negative effects of chemical production such as production of toxic byproducts and disposal of the chemical. The approach which strives to identify synthetic pathways that are less polluting than existing pathways and that encourages the development of nontoxic chemical products is referred to as "Green Chemistry".

Because the EPA functions in both regulatory and scientific modes, there is a need to foster greater understanding of risks associated with both conventional as well as novel technologies. Failure to do this can result in expensive and needless regulatory oversight, while prolonging the process of bringing "environmentally friendly" products online. This talk will provide a discussion of both risk assessment tools and some recent developments which have been brought to EPA's attention either through Agency funding or regulatory review. Among the topics that will be discussed are the following:

- development of a biotechnology risk assessment program with a focus on addressing technical issues that are growing more complex;

- substitution of microorganisms to manufacture adipic acid starting with microbial nutrients rather than benzene;

- construction of a microorganism which uses biological fluorescence to detect the presence of biologically available toxic materials;

- generation of a biomass conversion process which produces alcohol from biomass products.

The presentation will discuss recent developments in these fields as well as opportunity to better coordinate work in these areas between federal departments and agencies. The objective will be to leverage increasingly smaller resources to maximize benefits across the government, as well as presenting a more consistent approach to developmental and regulatory activities to nongovernmental agencies.

National Institutes of Health

NIGMS/NIDDK

The National Institute of General Medical Sciences (NIGMS), in conjunction with the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK), issued a program announcement in September 1995, in an effort to stimulate research in metabolic engineering. This announcement is part of a long-term, ongoing effort, and application for support of research in metabolic engineering are still being encouraged. Through this initiative, the NIH hopes to encourage basic research that will facilitate both the development of microbial or plant-base production routes for useful quantities of "small" molecules (such as antibiotics and other drugs) AND a substantially heightened

understanding of the control architecture that integrates the genetic and catalytic processes in normal and aberrant cells. During fiscal 1998, the NIGMS and NIDDK provided over $2.3 million for the support of research directly involving metabolic engineering. Examples of work funded through this initiative include (1) a study of the genes and enzymes which represent rate-limiting steps in the biosynthesis of beta-lactam antibiotics; (2) a study of the origin of bioactive marine natural products at the cellular level within selected deep water sponges; and (3) a study of the feasibility of genetically engineering fungi to produce novel polyketides with pharmacological potential.

National Science Foundation

The **Directorate of Engineering** supports several investigators in the area of metabolic engineering. One common feature of these research projects involves purposeful changes in organism behavior for increased product yields and levels for both wild type and recombinant systems. In addition, the improved biodegradation of toxic compounds is also being approached through metabolic engineering. Biological processes of this type have significant industrial potential, but in many cases still require the necessary biochemical engineering to translate them into a scalable process. In order to obtain the highest yields of metabolite products, restructuring of the central pathways for carbon catabolism and dispersal of incoming carbon into synthetic pathways will be necessary. Because of the tight integration among these pathways and the energy-producing pathways, restructuring of this central core of metabolism will require a systems approach which considers the interactions of the pathways concerned with the other metabolic subsystems in the cell. The system is complicated by regulation at both genetic and enzyme levels of all of these interacting metabolic subsystems. Therefore, an important aspect of the engineering research is the development of the mathematical systems, and control theory needed for a quantitative analysis and understanding of the metabolic changes which are initiated by the manipulation of the enzymatic, transport and regulatory functions of the cell. Examples of metabolic engineering research supported in this division include: (1) the use of linear optimization theory for the network analysis of intermediary metabolism, (2) the development of methods to select the internal fluxes for experimental measurement based on their sensitivity to experimental error, (3) the development of a method to determine flux control coefficients using transient metabolite concentrations, and (4) a study of network rigidity to help overcome the cell control mechanisms that resist flux alterations at branch points in metabolic pathways.

The **Directorate for Biological Sciences** (BIO) supports a broad range of research activities directed at increasing the knowledge base required for metabolic engineering. Examples of several BIO activities with implications for Metabolic Engineering include the following activities: (1) the "*Arabidopsis* Genome Research Initiative:" a multinational research cooperation to sequence the entire genome of the model plant, *Arabidopsis thaliana*, in order to establish baseline genomic data for plants, and to develop microarrays and other technology that can be used for further applications; (2) the "Plant Genome Research Program" which supports research on plant genome structure and function. Research supported by this program is characterized by a systems approach to plant genome research that builds upon recent advances in genomics, bioinformatics, and plant biology. This BIO activity has already funded 23 groups of investigators, often consortia of several universities and industries, to carry out sequencing and functional genomics projects. Supported efforts range from sequencing agriculturally important genomes (maize, soybean, tomato), to technology development, to focused applications (stress tolerance, pathogen responses, cotton fibers). (3) The "Microbial Observatories Initiative" a new initiative announced in November 1998, which includes the study of novel microorganisms in soils, marine sediments, and aquatic environments. The tremendous diversity of currently undescribed microorganisms offers potential metabolic engineering spin-offs such as new pathways for biodegradation of environmental toxins and novel pharmaceuticals. (4) The ongoing "Life in Extreme Environments (LEXEN) Initiative" also offers a strong link to Metabolic Engineering because of the wealth of metabolic pathways evolved by organisms that have adapted to environmental extremes. Also included in the LEXEN program are proposals to investigate the potential for habitable environments on other planets. (5) Finally, the BIO Directorate has recently announced the "2010 Project" that will support research to determine the function of all genes in *Arabidopsis thaliana* by the year

2010. The program will focus on supporting creative and innovative research designed to determine the function of a network of genes and to develop new tools for functional genomic approaches.

The **Directorate for Geosciences** supports research related to ME in marine systems. Research areas include: (1) determination of the physico-chemical requirements for the maintenance, growth, and regulation of marine microbes; (2) identification, isolation, and determination of the function of enzymes responsible for useful degradation processes; (3) exploration of marine viruses and how they can be used in genetic engineering; (4) development of molecular assays for harmful species of marine microbes; (5) determination of cellular and biochemical control of trace metal limitation; (6) characterization of enzymes and genes associated with nitrogen fixation in cyanobacteria; and (7) identification and characterization of marine microbes and consortia that degrade, detoxify, or metabolize marine pollutants.

The **Directorate for Mathematical and Physical Sciences** supports a number of projects involving metabolic engineering. Of particular interest is the generation of new enzymes to facilitate catalytic processes such as the desymmetrization of achiral molecules and the development of new bacterial strains that will be useful for the conversion of petrochemical and other industrial byproducts into useful or benign derivatives. Theoretical work continues to explore the basis of information encoding which is the foundation of molecular genetics and its associated properties of self-replication and the nonrandom organization of genetic material into specific shapes. Bridges to the experimental realm provide ever more elegant examples of synthetic structures that mimic genetic principles. These experiments are expanding our understanding of the underlying chemistry of genetic and biochemical processes and provide the basis for such functional examples of chemical systems patterned after living systems as enzyme mimics. Additionally, the increasing understanding of the specific ways that drug molecules interact with gene-derived entities is the basis of a new era of chemotherapy.

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